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L6: Entry 2 of 7 File: PGPB Oct 30, 2003

PGPUB-DOCUMENT-NUMBER: 20030202966
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030202966 A1

TITLE: Isolated stromal cells and methods of using the same

PUBLICATION-DATE: October 30, 2003

US-CL-CURRENT: 424/93.7; 435/366

APPL-NO: 10/ 423232 [PALM]
DATE FILED: April 25, 2003

RELATED-US-APPL-DATA:
Application 10/423232 is a continuation-of US application 08/913918, filed December 8, 1997, PENDING
Application 08/913918 is a continuation-of US application PC/T/US96/04407, filed March 28, 1996, PENDING
Application PC/T/US96/04407 is a continuation-in-part-of US application 08/412066, filed March 28, 1995, US Patent No. 5716616
Application is a non-provisional-of-provisional application 60/006627, filed November 13, 1995,

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L6: Entry 6 of 7 File: USPT Nov 25, 2003

US-PAT-NO: 6653134
DOCUMENT-IDENTIFIER: US 6653134 B2

TITLE: Isolated stromal cells for use in the treatment of diseases of the central nervous system

DATE-ISSUED: November 25, 2003

US-CL-CURRENT: 435/377; 435/366, 435/368, 435/372, 435/373

APPL-NO: 09/ 028395 [PALM]
DATE FILED: February 24, 1998

PARENT-CASE:
CROSS-REFERENCE TO RELATED APPLICATIONS The present application is a continuation-in-part of PCT Application No. PCT/US96/04407, filed on Mar. 28, 1996, which is a continuation of U.S. application Ser. No. 08/412,066, filed on Mar. 28, 1995, now U.S. Pat. No. 5,716,616, and which is also entitled to priority under 35 U.S.C. 119 (e) to U.S. Provisional Application No. 60/006,627, filed on Nov. 13, 1995.

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L6: Entry 6 of 7 File: USPT Nov 25, 2003

US-PAT-NO: 6653134
DOCUMENT-IDENTIFIER: US 6653134 B2

TITLE: Isolated stromal cells for use in the treatment of diseases of the central nervous system

DATE-ISSUED: November 25, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Prockop; Darwin J.	Philadelphia	PA		
Stokes; David G.	Willow Grove	PA		
Azizi; S. Ausim	Philadelphia	PA		

US-CL-CURRENT: 435/377; 435/366, 435/368, 435/372, 435/373

CLAIMS:

What is claimed is:

1. A method of directing the differentiation neural of an isolated stromal cell into a neural cell, comprising culturing said isolated stromal cell in the presence of a substantially homogeneous population of differentiated neural cells whereby said isolated stromal cell differentiates and acquires the phenotypic characteristics of said differentiated neural cells.

2. The method of claim 1, wherein said differentiated cells are astrocytes.

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L6: Entry 7 of 7 File: USPT Feb 10, 1998

US-PAT-NO: 5716616
DOCUMENT-IDENTIFIER: US 5716616 A

TITLE: Isolated stromal cells for treating diseases, disorders or conditions characterized by bone defects

DATE-ISSUED: February 10, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Prockop; Darwin J.	Philadelphia	PA		
Pereira; Ruth F.	Lansdowne	PA		
Leeper; Dennis B.	Wynnewood	PA		
O'Hara; Michael D.	Wyncote	PA		

US-CL-CURRENT: 424/93.7; 424/93.71, 424/93.72, 424/93.73

CLAIMS:

We claim:

1. A method of treating a patient who is suffering from a disease, disorder or condition characterized by a bone defect comprising the steps of:

a) obtaining a bone marrow sample from a donor who is not suffering from a disease, disorder or condition characterized by a bone defect;

b) isolating adherent cells from said sample; and,

c) administering by intravenous infusion to said patient an amount of said isolated adherent cells effective to treat said disease, disorder or condition, wherein said patient undergoes bone marrow ablation prior to administration of said isolated adherent cells.

2. The method of claim 1 wherein said adherent cells are administered by intravenous infusion to said patient together with non-adherent cells from a bone marrow sample from a donor who is not suffering from a disease, disorder or condition characterized by a bone defect.

3. The method of claim 1 wherein said disease, disorder or condition is osteogenesis imperfecta.

4. The method of claim 1 wherein said bone defect is due to a collagen gene mutation.

5. The method of claim 1 wherein said donor is syngeneic with said patient.

6. The method of claim 1 wherein said isolated adherent cells are cultured to expand the number of said cells and said expanded culture is administered to said individual.

7. The method of claim 6 wherein said disease, disorder or condition is osteogenesis imperfecta.

8. The method of claim 6 wherein said bone defect is due to a collagen gene mutation.

9. The method of claim 6 wherein said donor is syngeneic with said patient.

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L6: Entry 2 of 7 File: PGPB Oct 30, 2003

DOCUMENT-IDENTIFIER: US 20030202966 A1
TITLE: Isolated stromal cells and methods of using the same

Continuity Related Application Number:
5716616

Continuity Data:
parent-patent 5716616 US

Detail Description Paragraph:
[0050] According to the invention, isolated stromal cells are furnished with genetic material which renders them specifically susceptible to destruction. For example, the stromal cells may be provided with genes that encode a receptor that can be specifically targeted with a cytotoxic agent. An expressible form of a gene that can be used to induce selective cell death can introduced into the cells. In such a system, cells expressing the protein encoded by the gene are susceptible to targeted killing under specific conditions or in the presence or absence of specific agents. For example, an expressible form of a herpes virus thymidine kinase (herpes tk) gene can be introduced into the cells and used to induce selective cell death. When the exogenous genetic material that includes (herpes tk) gene is introduced into the individual, herpes tk will be produced. If it is desirable or necessary to kill the implanted cells, the drug gangcyclovir can be administered to the individual and that drug will cause the selective killing of any cell producing herpes tk. Thus, a system can be provided which allows for the selective destruction of implanted cells.

Detail Description Paragraph:
[0107] To establish producer cell lines, amphotrophic retrovirus packaging murine cells PA317 were used. The cells were transfected at 20% confluency in 100 mm dishes by the calcium phosphate precipitation procedure (Promega) using 15 .mu.g of plasmid DNA that was linearized by digestion with ScaI that cuts in the pBR322 region of the retrovirus vector. One day post-transfection G418 (GIBCO/BRL) was added to the medium at an active concentration of 1 mg/ml. Neomycin-resistant colonies appeared at 7 to 10 days of selection and were isolated by cloning with mechanical rings. The clones were expanded and individual clones were tested for the ability to express lacZ by direct staining of duplicate wells. The titer of the virus produced by the positive cells was assayed by single addition of 50 .mu.l of medium to HT-1080 human tumor cells grown to 20% confluency in 6-well microliter plates with 3 ml medium per well and in the presence of 4 .mu.g/ml of polybrene. The titer was assayed by determining the number of HT-1080 cells that stained positively for expression of the lacZ gene. Typically, the titer was 1.times.10.sup.5 to 1.times.10.sup.6.

Detail Description Paragraph:
[0111] Primary cultures of MSCs were grown for 10 days in (.alpha.-MEM containing 10% FBS). After trypsinization and light scraping, the cells were seeded in a 6-well plate at a density of 10.sup.5 cells per well. The cells were grown for 2 days, then washed 2 times with PBS and incubated with a DNA-lipofectamine complex. The DNA-lipofectamine complex was prepared as follows: 6 .mu.l of lipofectamine (GIBCO/BRL) were mixed with 1 .mu.g of LINCZ DNA in 200 .mu.l of .alpha.-MEM,

incubated at room temperature for 30 min, and added to one well of a 6-well plate containing MSCs in 800 .mu.l .alpha.-MEM. After 6 h incubation at 37.degree. C., the DNA-lipofectamine complex was replaced with 2 ml of .alpha.-MEM containing 10% FBS. The cells were stained for lacZ or placed under G418 selection after 18 h incubation in FBS-containing medium. Positive clones were obtained, but they grew slowly, apparently because the cell density was too low after the G418 selection. To circumvent this situation, three different strategies can be used: (a) cells are plated at higher densities; (b) co-culture cell culture inserts will be placed over surviving clones early in the selection process and place fresh MSCs or pieces of bone in the inserts (see Table 1) on a daily basis to provide the necessary cell factors to stimulate growth; (c) at the time that selection with G418 has killed many of the non-transfected calls, the cultures are reseeded with MSCs that have been infected with a variant of the retrovirus LNCX (FIG. 1) in which the lacZ gene is replaced with a selectable gene for thymidine kinase. Therefore, the MSCs stably transfected with retrovirus are used to provide the necessary cytokines, growth factors, and cell interactions required for the initial growth of the transfected MSCs during selection in G418. We can then remove the cells infected with the retrovirus by negative selection with gangcyclovir.

CLAIMS:

5. The method of claim 1 wherein prior to administering said stromal cells, said stromal cells are transfected with a gene construct that comprises a herpes thymidine kinase gene, wherein said gene is operably linked to regulatory sequences and is expressed by said stromal cells.

15. The method of claim 12 wherein prior to administering said stromal cells, said stromal cells are transfected with a gene construct that comprises a herpes thymidine kinase gene, wherein said gene is operably linked to regulatory sequences and is expressed by said stromal cells.

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L10: Entry 5 of 114 File: PGPB Nov 27, 2003

DOCUMENT-IDENTIFIER: US 20030219441 A1
TITLE: Combined methods and compositions for coagulation and tumor treatment

Detail Description Paragraph:
[0592] Exemplary tTF prodrugs have the following structures: tTF.sub.1-219 (X).sub.n1 (Y).sub.n2 Z Ligand, where tTF.sub.1-219 represents TF minus the cytosolic and transmembrane domains; X represents a hydrophobic transmembrane domain n1 amino acids (AA) in length (1-20 AA); Y represents a hydrophilic protease recognition sequence of n2 AA in length (sufficient AA to ensure appropriate protease recognition); Z represents a disulfide thioester or other linking group such as (Cys).sub.1-2; Ligand represents an antibody or other targeting moiety recognizing tumor-cells, tumor EC, connective tissue (stroma) or basal lamina markers

Detail Description Paragraph:
[0844] Lord et al., In: Genetically Engineered Toxins, Frank (Ed.), M. Dekker Publ., p. 183, 1992.

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L10: Entry 57 of 114 File: PGPB Mar 14, 2002

PGPUB-DOCUMENT-NUMBER: 20020031824
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020031824 A1

TITLE: Method for homing hematopoietic stem cells to bone marrow stromal cells

PUBLICATION-DATE: March 14, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Greenberger, Joel S.	Lincoln	MA	US	

US-CL-CURRENT: 435/372; 424/93.21

CLAIMS:

1. A method for homing hematopoietic stem cells to bone marrow stromal cells in a host, comprising: administering to the host genetically-engineered hematopoietic stem cells capable of expressing a first member of a ligand-receptor binding pair under conditions whereby binding of the first member of the ligand-receptor binding pair to a second member of the ligand-receptor binding pair, present on stromal cells, occurs thereby homing the stem cells to the stromal cells.

2. A method for homing hematopoietic stem cells to bone marrow stromal cells in a host, comprising: administering to the host stromal cells capable of expressing a first member of a ligand-receptor binding pair; and subsequently administering to the host hematopoietic stem cells capable of expressing a second member of a ligand-receptor binding pair under conditions whereby binding of the ligand to the receptor occurs thereby homing the stem cells to the stromal cells, wherein either the stromal cells or the hematopoietic stem cells are genetically-engineered to provide the capability of expressing the appropriate ligand or receptor.

3. A method according to claim 2 wherein the ligand-receptor binding pair is selected from the group consisting transforming growth factor and transforming growth factor receptor or epidermal growth factor receptor; epidermal growth factor and epidermal growth factor receptor; tumor necrosis factor-.alpha. and tumor necrosis factor-receptor; interferon and interferon receptor; platelet derived growth factor and platelet derived growth factor receptor; transferrin and transferrin receptor; avidin and biotin or antibiotin; antibody and antigen pairs; interleukin and interleukin receptor; granulocyte-macrophage colony stimulating factor and granulocyte-macrophage colony receptor; macrophage colony stimulating factor and macrophage colony stimulating factor receptor; granulocyte colony stimulating factor and granulocyte colony stimulating factor receptor; and sugar molecules and sugar receptors.

4. A method according to claim 2 wherein the hematopoietic stem cells are 32D or a non-adherent stem cell derived from a human long term bone marrow culture.

5. A method according to claim 2 wherein the bone marrow stromal cells are selected from the group consisting of GBL/6, KM101, KM102, KM103, KM104 and K105.

6. A method according to claim 2 wherein the stromal cells are capable of expressing a ligand.

7. A method according to claim 6 wherein the ligand is pro-transforming growth factor-.alpha..

8. A method according to claim 2 wherein the hematopoietic stem cells are capable of expressing a receptor.

9. A method according to claim 8 wherein the receptor is an epidermal growth factor receptor.

10. A method according to claim 2 wherein either the genetically-engineered stromal cells or hematopoietic stem cells are produced by transfecting the cells with a retroviral vector containing RNA which is reverse transcribed to DNA encoding a member of a ligand-receptor binding pair.

11. A method for transplanting bone marrow in a host, comprising: administering to the host stromal cells capable of expressing a first member of a ligand-receptor binding pair; and administering to the host hematopoietic stem cells capable of expressing a second member of a ligand-receptor binding pair under conditions whereby binding of the ligand to the receptor occurs thereby homing the stem cells to the stromal cells, wherein either the stromal cells or hematopoietic stem cells are genetically-engineered to provide the capability of expressing the appropriate ligand or receptor.

12. A method according to claim 11 wherein the ligand is pro-transforming growth factor-.alpha. and the receptor is an epidermal growth factor receptor.

13. A method according to claim 11 wherein the hematopoietic stem cells are selected from the group consisting of 32D or a non-adherent stem cell derived from a human long term bone marrow culture.

14. A method according to claim 11 wherein the bone marrow stromal cells are selected from the group consisting of GBL/6, KM101, KM102, KM103, KM104 and KM105.

15. A method according to claim 11 wherein the stromal cells are capable of expressing a ligand.

16. A method according to claim 15 wherein the ligand is transforming growth factor-.alpha..

17. A method according to claim 11 wherein the genetically-engineered hematopoietic stem cells are capable of expressing a receptor.

18. A method according to claim 17 wherein the receptor is an epidermal growth factor receptor.

19. A method according to claim 11 wherein the genetically-engineered stromal cells or the hematopoietic stem cells are produced by transfecting the cells with a retroviral vector containing RNA which is reverse transcribed to DNA encoding a member of a ligand-receptor binding pair.

20. A method of treating a host afflicted with a disease associated with a disorder of the bone marrow, comprising: administering to the host a therapeutically effective amount of stromal cells capable of expressing a first member of a ligand-receptor binding pair; and administering to the host a therapeutically effective amount of hematopoietic stem cells capable of expressing a second member of a ligand-receptor binding pair under conditions whereby binding of the ligand to the receptor occurs, thereby homing the stem cells to the stromal cells, wherein either the stromal cells or hematopoietic stem cells are genetically-engineered to provide the capability of expressing the appropriate ligand or receptor.

L10: Entry 104 of 114 File: USPT Sep 9, 1997

DOCUMENT-IDENTIFIER: US 5665557 A
** See image for Certificate of Correction **
TITLE: Method of purifying a population of cells enriched for hematopoietic stem cells populations of cells obtained thereby and methods of use thereof

Brief Summary Text (11):
Stem cells are also important targets for gene therapy, where expression of the inserted genes promotes the health of the individual into whom the stem cells are transplanted. In addition, the ability to isolate stem cells may serve in the treatment of lymphomas and leukemias, as well as other neoplastic conditions where the stem cells are purified from tumor cells in the bone marrow or peripheral blood, and reinfused into a patient after myelosuppressive or myeloablative chemotherapy. Thus, there have been world-wide efforts toward isolating stem cells in substantially pure or pure form.

Drawing Description Text (45):
Once the CDw109.sup.+ cells have been isolated, they may be propagated on stromal cells, such as stromal cells that can be obtained from bone marrow, fetal thymus or fetal liver, and are shown to provide for the secretion of growth factors associated with progenitor cell maintenance where the stromal cells may be allogeneic or xenogeneic. Before using in the co-culture, the mixed stromal cell preparations may be freed of hematopoietic cells employing appropriate monoclonal antibodies for removal of the undesired cells, e.g., with antibody-toxin conjugates, antibody and complement, etc. Alternatively, cloned stromal cell lines may be used where the stromal lines may be allogeneic or xenogeneic.

Drawing Description Text (47):
The CDw109.sup.+ cells may be used in gene therapy for the treatment of a variety of diseases, particularly genetic diseases. Genetic diseases associated with hematopoietic cells may be treated by genetic modification of autologous or allogeneic stem cells to correct the genetic defect. For example, diseases including, but not limited to, .beta.-thalassemia, sickle cell anemia, adenosine deaminase deficiency, recombinase deficiency, recombinase regulatory gene deficiency, etc. may be corrected by introduction of a wild-type gene into the CDw109.sup.+ cells, either by homologous or random recombination. Other indications of gene therapy are introduction of drug resistance genes to enable normal stem cells to have an advantage and be subject to selective pressure during chemotherapy. Suitable drug resistance genes include, but are not limited to, the gene encoding the multidrug resistance (MDR) protein.

Drawing Description Text (49):
Alternatively, one may wish to remove a particular variable region of a T-cell receptor from the T-cell repertoire. By employing homologous recombination, or antisense or ribozyme sequence which prevents expression, the expression of the particular T-cell receptor may be inhibited. For hematotrophic pathogens, such as HIV, HTLV-I and II, etc. the stem cells could be genetically modified to introduce an antisense sequence or ribozyme which would prevents the proliferation of the pathogen in the stem cell or cells differentiated from the stem cells. Methods for recombination in mammalian cells may be found in Molecular Cloning, A Laboratory Manual (1989) Sambrook, Fritsch and Maniatis, Cold Spring Harbor, N.Y.

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L10: Entry 110 of 114 File: USPT Feb 11, 1992

DOCUMENT-IDENTIFIER: US 5087570 A
TITLE: Homogeneous mammalian hematopoietic stem cell composition

Detailed Description Text (19):
Once stem cells have been isolated, they may be propagated by growing in conditioned medium from stromal cells, such as those that can be obtained from bone marrow or liver associated with the secretion of factors, or in medium comprising cell surface factors supporting the proliferation of stem cells. Stromal cells may be freed of hematopoietic cells employing appropriate monoclonal antibodies for removal of the undesired cells, for example, with antibody-toxin conjugates, antibody and complement, etc.

CLAIMS:

1. A cell composition consisting essentially of as the cellular population viable murine hematopoietic Sca-1+ stem cells from a genetically identical or congenic source.

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L13: Entry 17 of 17 File: USPT Jan 7, 1997

DOCUMENT-IDENTIFIER: US 5591625 A
TITLE: Transduced mesenchymal stem cells

Abstract Text (1):
Genetically engineered human stem cells that carry within them genes of interest particularly for the expression of physiologically or pharmacologically active proteins or for use in gene therapy. In addition to correction of genetic disorders, is the ability to introduce, in a targeted manner, additional copies of essential genes to allow expression in proliferating, nondifferentiating cells of certain gene products. These genes can be, for example, hormones matrix proteins, cytokines, adhesion molecules, detoxification enzymes and "rebuilding" proteins important in tissue repair.

Brief Summary Text (1):
The present invention is directed to a totally unexplored application of human stem cells, i.e. that of genetically engineered cell that carry within them genes of interest particularly for the expression of physiologically or pharmacologically active proteins or for use in gene therapy.

Brief Summary Text (10):
In one embodiment, the isolated human stem cells are preferably mesenchymal stem cells that have been transformed with at least one DNA sequence capable of expressing those translation products capable of packaging a viral sequence so as to be gene therapy producer cells. In a preferred embodiment of this aspect, the isolated human stem cells have been transformed with a DNA sequence comprising a retroviral 5' LTR and, under the transcriptional control thereof, at least one of a retroviral gag, pol or env gene. In another aspect, the isolated human stem cells have also been transformed with a DNA sequence comprising a retroviral packaging signal sequence and incorporated genetic material to be expressed under the control of a promoter therefor so as to be incompetent retroviruses. Also contemplated is the transfection of MSCs or committed stromoblasts to initiate, modulate or augment hematopoiesis.

Brief Summary Text (13):
The advantages provided by the present invention include (a) the ability to culturally expand human stem cells for (re)infusion where they will localize to mesenchymal tissue spaces; (b) the ability to culturally expand and cryopreserve human mesenchymal stem cells which can be used as hosts for stable, heritable gene transfer; (c) the ability to recover genetically altered cells after installation in vivo; (d) the ability to match a genetic therapy to a wide variety of disorders, pinpointing the genetic alteration to the target tissue; and (e) the ability of newly introduced genes within human stem cells and their progeny to be expressed in a less restrictive fashion than other cells, thereby expanding the potential application in treating medical disease.

Brief Summary Text (36):
It is also possible to use vehicles other than retroviruses to genetically engineer or modify stem cells. Genetic information of interest can be introduced by means of

any virus which can express the new genetic material in such cells. For example, SV40, herpes virus, adenovirus and human papilloma virus can be used for this purpose. Many other methods can also be used for introducing cloned eukaryotic DNAs into cultured mammalian cells, several of which are discussed below. The genetic material to be transferred to stem cells may be in the form of viral nucleic acids, bacterial plasmids or episomes. The latter have the advantage of extracellular nephication both in vitro and in vivo.

Brief Summary Text (43):
The present invention makes it possible to genetically engineer mesenchymal human stem cells in such a manner that they produce polypeptides, hormones and proteins not normally produced in human stem cells in biologically significant amounts or produced in small amounts but in situations in which overproduction would lead to a therapeutic benefit. These products would then be secreted into the bloodstream or other areas of the body, such as the central nervous system. The human stem cells formed in this way can serve as a continuous drug delivery systems to replace present regimens, which require periodic administration (by ingestion, injection, depot infusion etc.) of the needed substance.

Brief Summary Text (45):
Genetically engineered human mesenchymal stem cells can also be used for the production of clotting factors. Hemophiliacs lack a protein called Factor VIII, which is involved in clotting. Factor VIII is now administered by injection. Human stem cells having genes encoding Factor VIII, can be used to make a skin graft (human MSCs are present in the dermis) in which they produce Factor VIII; as a skin graft, the tissue secretes the factor into the bloodstream. Such cells can also be used for continuous delivery of dystrophin to muscle cells fro muscular dystrophy.

Brief Summary Text (46):
Incorporation of genetic material of interest into human stem cells and other types of cells is particularly valuable in the treatment of inherited and acquired disease. In the case of inherited diseases, this approach is used to provide genetically modified human stem cells and other cells which can be used as a metabolic sink. That is, such human stem cells would serve to degrade a potentially toxic substance. For example, this could be used in treating disorders of amino acid catabolism including the hyperphenylalaninemias, due to a defect in phenylalanine hydroxylase; the homocysteinemias, due to a defect in cystathionine .beta.-synthase. Other disorders that could be treated in this way include disorders of amino acid metabolism, such as cystinosis; disorders of membrane transport, such as histidinurea or familial hypecholesterolemia; and disorders of nucleic acid metabolism, such as hereditary orotic aciduria. Human mesenchymal stem cells of the present invention can also be used in the treatment of genetic diseases in which a product (e.g., an enzyme or hormone) normally produced by the body is not produced or is made in insufficient quantities. Here, human stem cells transduced with a gene encoding the missing or inadequately produced substance can be used to produce it in sufficient quantities. This can be used in producing alpha-1 antitrypsin. It can also be used in the production of Factor XIII and Factor IX and thus would be useful in treating hemophilia. For any of these examples, includes in the present invention is the use of tissue specific promoters that allow increased expression in particular mesenchymal cell lineages and cells which would be used to limit gene expression into either the differentiated or precursor stem cell. Examples of such tissue-specific promoters include but are not limited to the promoter for the collagen type I genes or another collagen gene family, the promoter for the dystrophin gene and the promoter for stem cell factor.

Brief Summary Text (49):
Another application is a subcutaneous implatation of stem cells alone or adhered to a porous ceramic cube device which will house the stem cells and allow them to differentiate in vivo. Another example would be injection of stem cells into muscle

where they will differentiate into muscle cells. An example might be a graft having genetically engineered human stem cells is in birth control. Tests are underway now for using a polypeptide hormone called lutenizing hormone releasing hormone (LHRH) in regulating fertility. Continuous administration of LHRH results in a sterile individual; when administration ceases, the individual is again fertile. Rather than taking LHRH injections or oral medication, one could have a small graft which continuously secretes LHRM to provide the same effect. In the event that the person wanted to regain fertility this transplant could be excised; delivery of the polypeptide hormone would cease.

Brief Summary Text (58):
6. cytotoxic genes such as thymidine kinase which sensitizes cells to ganciclovir. Gap junction adhesion to tumor cells could allow MSCs to serve for cancer therapy.

Detailed Description Text (24):
These results indicated: a) that the precursor, genetically transduced MSCs expressed both the neo and lacZ genes when grown under selection pressure of G-418 in vitro; b) that these retrovirally transduced cells retained their "stem cell" phenotype, after in vitro passage for many weeks without evidence of differentiation into osteogenic cells or stromal bone marrow cells, as measured by their ability to differentiate into osteoblasts and osteocytes in vivo, and c) that even when no longer under the selection pressure of G418, these cells retained the ability to express a foreign gene in vivo for at least 8 weeks as they proliferate and pass through the differentiation process, i.e. the genetic transduction has become a stable part of the cellular, genomic DNA. As such, they are unique in being human mesenchymal stem cells derived from a non-fetal, or in this case, adult host which have the capacity to be transduced and culture expanded and have been shown to retain their precursor stem cell phenotype. While loss of the transduced genes occurred in some cells, the majority of cells appear to have retained the proviral genes after a period of prolonged growth in vivo.

Other Reference Publication (1):
F. D. Ledley (1991) Human Gene Therapy 2:77-83.

Other Reference Publication (49):
Anderson, Human Gene Therapy, 1:331-362 (1990).

Other Reference Publication (59):
Beck-Engeser et al., Human Gene Therapy, 2:61-70 (1991).

Other Reference Publication (60):
Ledley, Human Gene Therapy, 2:77-83 (1991).

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L28: Entry 471 of 570 File: USPT May 4, 1999

US-PAT-NO: 5899936
DOCUMENT-IDENTIFIER: US 5899936 A

TITLE: Treated tissue for implantation and methods of preparation

DATE-ISSUED: May 4, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goldstein; Steven	Atlanta	GA		

US-CL-CURRENT: 128/898; 435/371, 623/23.72, 623/920, 623/921

CLAIMS:

What is claimed is:

1. A process for treating bodily tissue which has been harvested from a donor to improve its compatibility with the immune system of an implant recipient to which said tissue in untreated form is xenogeneic, comprising the steps of

treating said bodily tissue to form a decellularized tissue matrix;

applying to a decellularized tissue matrix an amount of one or more cellular adhesion factors effective to promote the subsequent adhesion of cultured cells within the tissue matrix, wherein the cellular adhesion factor comprises one or more extracellular proteins ordinarily associated with the tissue in a liquid vehicle;

and then repopulating the tissue matrix with cells which are autologous or allogeneic to said recipient to provide a substantially non-immunogenic and biomechanically acceptable implant or graft which is vitalized by the cellular repopulation and is otherwise histologically and biochemically similar to the untreated tissue.

2. The process of claim 1 wherein the tissue treated is connective tissue, heart tissue or collagenous or vascular tissue.

3. The process of claim 2 wherein the adhesion factors include a glycoprotein and a glycosaminoglycan.

4. The process of claim 3 wherein the tissue matrix is repopulated with fibroblast cells immunologically compatible with the implant recipient.

5. The process of claim 4 wherein the fibroblast cells are modified genetically by techniques of

stable transfection with exogenous genetic material.

6. The process of claim 4 wherein the fibroblast cells are of human origin and are modified to be substantially non-immunogenic upon implantation.

7. The process of claim 4 wherein the fibroblast cells are of human origin and are modified by genetic manipulation to express specific proteins.

8. The process of claim 4 wherein the adhesion factor is comprised of fibronectin and a glycosaminoglycan selected from the group consisting of dermatin, dermatin sulfate, chondroitin, chondroitin sulfate, heparin sulfate and heparin.

9. The process of claim 8 wherein the tissue is porcine heart valve tissue, and wherein the cellular repopulation step is conducted by incubating the tissue matrix in a nutrient environment and in the presence of fibroblast cells and an effective amount of fibroblast growth factor.

L28: Entry 484 of 570 File: USPT Jan 12, 1999

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TITLE: Three-dimensional cell and tissue culture system

Abstract Text (1):
The present invention relates to a three-dimensional cell culture system which can be used to culture a variety of different cells and tissues in vitro for prolonged periods of time. In accordance with the invention, cells derived from a desired tissue are inoculated and grown on a pre-established stromal support matrix. The stromal support matrix comprises stromal cells, such as fibroblasts actively growing on a three-dimensional matrix. Stromal cells may also include other cells found in loose connective tissue such as endothelial cells, macrophages/monocytes, adipocytes, pericytes, reticular cells found in bone marrow stroma, etc. The stromal matrix provides the support, growth factors, and regulatory factors necessary to sustain long-term active proliferation of cells in culture. When grown in this three-dimensional system, the proliferating cells mature and segregate properly to form components of adult tissues analogous to counterparts found in vivo.

Brief Summary Text (8):
While the growth of cells in two dimensions is a convenient method for preparing, observing and studying cells in culture, allowing a high rate of cell proliferation, it lacks the cell-cell and cell-matrix interactions characteristic of whole tissue in vivo. In order to study such functional and morphological interactions, a few investigators have explored the use of three-dimensional substrates such as collagen gel (Douglas et al., 1980, In Vitro 16:306-312; Yang et al., 1979, Proc. Natl. Acad. Sci. 76:3401; Yang et al., 1980, Proc. Natl. Acad. Sci. 77:2088-2092; Yang et al., 1981, Cancer Res. 41:1021-1027); cellulose sponge, alone (Leighton et al., 1951, J. Natl. Cancer Inst. 12:545-561) or collagen coated (Leighton et al., 1968, Cancer Res. 28:286-296); a gelatin sponge, Gelfoam (Sorour et al., 1975, J. Neurosurg. 43:742-749).

Brief Summary Text (9):
In general, these three-dimensional substrates are inoculated with the cells to be cultured. Many of the cell types have been reported to penetrate the matrix and establish a "tissue-like" histology. For example, three dimensional collagen gels have been utilized to culture breast epithelium (Yang et al., 1981, Cancer Res. 41:1021-1027) and sympathetic neurons (Ebendal, 1976, Exp. Cell Res. 98:159-169). Additionally, various attempts have been made to regenerate tissue-like architecture from dispersed monolayer cultures. Kruse and Miedema (1965, J. Cell Biol. 27:273) reported that perfused monolayers could grow to more than ten cells deep and organoid structures can develop in multilayered cultures if kept supplied with appropriate medium (see also Schneider et al., 1963, Exp. Cell Res. 30:449-459 and Bell et al., 1979, Proc. Natl. Acad. Sci. USA 76:1274-1279); Green (1978, Science 200:1385-1388) has reported that human epidermal kerotinocytes may form dematoglyphs (friction ridges) if kept for several weeks without transfer; Folkman and Haudenschild (1980, Nature 288:551-556) reported the formation of capillary tubules in cultures of vascular endothelial cells cultured in the presence of endothelial growth factor and medium conditioned by tumor cells; and Sirica et al. (1979, Proc. Natl. Acad. Sci. U.S.A. 76:283-287; 1980, Cancer Res. 40:3259-3267) maintained hepatocytes in primary culture for about 10-13 days on nylon meshes

coated with a thin layer of collagen. However, the long term culture and proliferation of cells in such systems has not been achieved.

Brief Summary Text (12):
The present invention relates to a three-dimensional cell culture system which can be used to culture a variety of different cells and tissues in vitro for prolonged periods of time. In accordance with the invention, cells derived from a desired tissue are inoculated and grown on a pre-established stromal support matrix. The stromal support matrix comprises stromal cells, such as fibroblasts, actively growing on a three-dimensional matrix. Stromal cells may also include other cells found in loose connective tissue such as endothelial cells, macrophages/monocytes, adipocytes, pericytes, reticular cells found in bone marrow stroma, etc. The stromal matrix provides the support, growth factors, and regulatory factors necessary to sustain long-term active proliferation of cells in culture. When grown in this three-dimensional system, the proliferating cells mature and segregate properly to form components of adult tissues analogous to counterparts found in vivo.

Brief Summary Text (13):
The invention is based, in part, on the discovery that growth of stromal cells in three dimensions will sustain active proliferation of cells in culture for longer periods of time than will monolayer systems. This may be due, in part, to the increased surface area of the three-dimensional matrix which results in a prolonged period of active proliferation of stromal cells. These proliferating stromal cells elaborate proteins, growth factors and regulatory factors necessary to support the long term proliferation of both stromal and tissue-specific cells inoculated onto the stromal matrix. In addition, the three-dimensionality of the matrix allows for a spatial distribution which more closely approximates conditions in vivo, thus allowing for the formation of microenvironments conducive to cellular maturation and migration. The growth of cells in the presence of this support may be further enhanced by adding proteins, glycoproteins, glycosaminoglycans, a cellular matrix, and other materials to the support itself or by coating the support with these materials.

Brief Summary Text (22):
Three-Dimensional Matrix: a three dimensional matrix composed of any material and/or shape that (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. This support is inoculated with stromal cells to form the three-dimensional stromal matrix.

Brief Summary Text (23):
Three-Dimensional Stromal Matrix: a three dimensional matrix which has been inoculated with stromal cells. Whether confluent or subconfluent, stromal cells according to the invention continue to grow and divide. The stromal matrix will support the growth of tissue-specific cells later inoculated to form the three dimensional cell culture.

Brief Summary Text (24):
Three-Dimensional Cell Culture: a three dimensional stromal matrix which has been inoculated with tissue-specific cells and cultured. In general, the tissue specific cells used to inoculate the three-dimensional stromal matrix should include the "stem" cells (or "reserve" cells) for that tissue; i.e., those cells which generate new cells that will mature into the specialized cells that form the parenchyma of the tissue.

Drawing Description Text (6):
FIG. 5 is a diagrammatic representation of the three-dimensional skin model. A dermal/epidermal junction is present, above which lies pigmented melanocytes and several layers of pigment-containing keratinocytes. The stromal cells attach to the

matrix and form the dermal component.

Detailed Description Text (2):
The present invention involves a three-dimensional matrix and its use as the framework for a three-dimensional, multi-layer cell culture system. In previously known tissue culture systems, the cells were grown in a monolayer. Cells grown on a three-dimensional stromal support, in accordance with the present invention, grow in multiple layers, forming a cellular matrix. This matrix system approaches physiologic conditions found in vivo to a greater degree than previously described monolayer tissue culture systems. The three-dimensional cell culture system is applicable to the proliferation of different types of cells and formation of a number of different tissues, including but not limited to bone marrow, skin, liver, pancreas, kidney, adrenal and neurologic tissue, to name but a few.

Detailed Description Text (4):
In accordance with the invention, cells derived from a desired tissue (herein referred to as tissue-specific cells or parenchymal cells) are inoculated and cultured on a pre-established three-dimensional stromal matrix. The stromal matrix comprises stromal cells grown on a three-dimensional matrix or network. The stromal cells comprise fibroblasts with or without additional cells and/or elements described more fully herein. The fibroblasts and other cells and/or elements that comprise the stroma may be fetal or adult in origin, and may be derived from convenient sources such as skin, liver, pancreas, etc. Such tissues and/or organs can be obtained by appropriate biopsy or upon autopsy. In fact, cadaver organs may be used to provide a generous supply of stromal cells and elements.

Detailed Description Text (5):
Fetal fibroblasts will support the growth of many different cells and tissues in the three-dimensional culture system, and, therefore, can be inoculated onto the matrix to form a "generic" stromal support matrix for culturing any of a variety of cells and tissues. However, in certain instances, it may be preferable to use a "specific" rather than "generic" stromal support matrix, in which case stromal cells and elements can be obtained from a particular tissue, organ, or individual. For example, where the three-dimensional culture is to be used for purposes of transplantation or implantation in vivo, it may be preferable to obtain the stromal cells and elements from the individual who is to receive the transplant or implant. This approach might be especially advantageous where immunological rejection of the transplant and/or graft versus host disease is likely. Moreover, fibroblasts and other stromal cells and/or elements may be derived from the same type of tissue to be cultured in the three-dimensional system. This might be advantageous when culturing tissues in which specialized stromal cells may play particular structural/functional roles; e.g., glial cells of neurological tissue, Kupffer cells of liver, etc.

Detailed Description Text (6):
Once inoculated onto the three-dimensional matrix, the stromal cells will proliferate on the matrix and support the growth of tissue-specific cells inoculated into the three-dimensional culture system of the invention. In fact, when inoculated with the tissue-specific cells, the three-dimensional stromal support matrix will sustain active proliferation of the culture for long periods of time. Growth and regulatory factors may be added to the culture, but are not necessary since they are elaborated by the stromal support matrix.

Detailed Description Text (7):
Because, according to the invention, it is important to recreate, in culture, the cellular microenvironment found in vivo for a particular tissue, the extent to which the stromal cells are grown prior to inoculation of parenchymal cells may vary depending on the type of tissue to be grown in three-dimensional tissue culture. For example, in bone marrow three-dimensional cultures, it is preferable to inoculate hematopoietic cells onto a stromal matrix which is subconfluent.

However, in skin three-dimensional tissue cultures, it is preferred, according to the invention, to allow the stromal cells to reach confluence prior to inoculation with keratinocytes and/or melanocytes, so as to recreate the structure of the dermal component of skin. Importantly, because openings in the mesh permit the exit of stromal cells in culture, confluent stromal cultures do not exhibit contact inhibition, and the stromal cells continue to grow, divide, and remain functionally active.

Detailed Description Text (10):
(a) The three-dimensional matrix provides a greater surface area for protein attachment, and consequently, for the adherence of stromal cells.

Detailed Description Text (11):
(b) Because of the three-dimensionality of the matrix, stromal cells continue to actively grow, in contrast to cells in monolayer cultures, which grow to confluence, exhibit contact inhibition, and cease to grow and divide. The elaboration of growth and regulatory factors by replicating stromal cells may be partially responsible for stimulating proliferation and regulating differentiation of cells in culture.

Detailed Description Text (17):
5.1. Establishment of Three-Dimensional Stromal Matrix

Detailed Description Text (18):
The three-dimensional support may be of any material and/or shape that: (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. A number of different materials may be used to form the matrix, including but not limited to: nylon (polyamides), dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (e.g., polyvinylchloride), polycarbonate (PVC), polytetrafluorethylene (PTFE; teflon), thermanox (TPX), nitrocellulose, cotton, polyglycolic acid (PGA), cat gut sutures, cellulose, gelatin, dextran, etc. Any of these materials may be woven into a mesh, for example, to form the three-dimensional matrix. Certain materials, such as nylon, polystyrene, etc., are poor substrates for cellular attachment. When these materials are used as the three-dimensional support matrix, it is advisable to pre-treat the matrix prior to inoculation of stromal cells in order to enhance the attachment of stromal cells to the matrix. For example, prior to inoculation with stromal cells, nylon matrices could be treated with 0.1M acetic acid, and incubated in polylysine, FBS, and/or collagen to coat the nylon. Polystyrene could be similarly treated using sulfuric acid.

Detailed Description Text (20):
Stromal cells comprising fibroblasts, with or without other cells and elements described below, are inoculated onto the matrix. These fibroblasts may be derived from organs, such as skin, liver, pancreas, etc. which can be obtained by biopsy (where appropriate) or upon autopsy. In fact fibroblasts can be obtained in quantity rather conveniently from any appropriate cadaver organ. As previously explained, fetal fibroblasts can be used to form a "generic" three-dimensional stromal matrix that will support the growth of a variety of different cells and/or tissues. However, a "specific" stromal matrix may be prepared by inoculating the three-dimensional matrix with fibroblasts derived from the same type of tissue to be cultured and/or from a particular individual who is later to receive the cells and/or tissues grown in culture in accordance with the three-dimensional system of the invention.

Detailed Description Text (23):
The isolation of fibroblasts may, for example, be carried out as follows: fresh tissue samples are thoroughly washed and minced in Hanks balanced salt solution (HBSS) in order to remove serum. The minced tissue is incubated from 1-12 hours in a freshly prepared solution of a dissociating enzyme such as trypsin. After such

incubation, the dissociated cells are suspended, pelleted by centrifugation and plated onto culture dishes. All fibroblasts will attach before other cells, therefore, appropriate stromal cells can be selectively isolated and grown. The isolated fibroblasts can then be grown to confluency, lifted from the confluent culture and inoculated onto the three-dimensional matrix (see, Naughton et al., 1987, J. Med. 18(3&4):219-250). Inoculation of the three-dimensional matrix with a high concentration of stromal cells, e.g., approximately 10.sup.6 to 5.times.10.sup.7 cells/ml, will result in the establishment of the three-dimensional stromal support in shorter periods of time.

Detailed Description Text (24):
In addition to fibroblasts, other cells may be added to form the three-dimensional stromal matrix required to support long term growth in culture. For example, other cells found in loose connective tissue may be inoculated onto the three-dimensional support along with fibroblasts. Such cells include but are not limited to endothelial cells, pericytes, macrophages, monocytes, plasma cells, mast cells, adipocytes, etc. These stromal cells may readily be derived from appropriate organs such as skin, liver, etc., using methods known in the art such as those discussed above. In one embodiment of the invention, stromal cells which are specialized for the particular tissue to be cultured may be added to the fibroblast stroma. For example, stromal cells of hematopoietic tissue, including but not limited to fibroblasts, endothelial cells, macrophages/monocytes, adipocytes and reticular cells, could be used to form the three-dimensional subconfluent stroma for the long term culture of bone marrow in vitro. Hematopoietic stromal cells may be readily obtained from the "buffy coat" formed in bone marrow suspensions by centrifugation at low forces, e.g., 3000.times.g. Stromal cells of liver may include fibroblasts, Kupffer cells, and vascular and bile duct endothelial cells. Similarly, glial cells could be used as the stroma to support the proliferation of neurological cells and tissues; glial cells for this purpose can be obtained by trypsinization or collagenase digestion of embryonic or adult brian (Ponten and Westermark, 1980, in Federof, S. Hertz, L., eds, "Advances in Cellular Neurobiology," Vol.1, New York, Academic Press, pp.209-227).

Detailed Description Text (25):
Again, where the cultured cells are to be used for transplantation or implantation in vivo it is preferable to obtain the stromal cells from the patient's own tissues. The growth of cells in the presence of the three-dimensional stromal support matrix may be further enhanced by adding to the matrix, or coating the matrix support with proteins (e.g., collagens, elastic fibers, reticular fibers) glycoproteins, glycosaminoglycans (e.g., heparan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, etc.), a cellular matrix, and/or other materials.

Detailed Description Text (26):
After inoculation of the stromal cells, the three-dimensional matrix should be incubated in an appropriate nutrient medium. Many commercially available media such as RPMI 1640, Fisher's, Iscove's, McCoy's, and the like may be suitable for use. It is important that the three-dimensional stromal matrix be suspended or floated in the medium during the incubation period in order to maximize proliferative activity. In addition, the culture should be "fed" periodically to remove the spent media, depopulate released cells, and add fresh media.

Detailed Description Text (27):
During the incubation period, the stromal cells will grow linearly along and envelop the three-dimensional matrix before beginning to grow into the openings of the matrix. It is important to grow the cells to an appropriate degree which reflects the amount of stromal cells present in the in vivo tissue prior to inoculation of the stromal matrix with the tissue-specific cells.

Detailed Description Text (28):

The openings of the matrix should be of an appropriate size to allow the stromal cells to stretch across the openings. Maintaining actively growing stromal cells which stretch across the matrix enhances the production of growth factors which are elaborated by the stromal cells, and hence will support long term cultures. For example, if the openings are too small, the stromal cells may rapidly achieve confluence but be unable to easily exit from the mesh; trapped cells may exhibit contact inhibition and cease production of the appropriate factors necessary to support proliferation and maintain long term cultures. If the openings are too large, the stromal cells may be unable to stretch across the opening; this will also decrease stromal cell production of the appropriate factors necessary to support proliferation and maintain long term cultures. When using a mesh type of matrix, as exemplified herein, we have found that openings ranging from about 150 .mu.m to about 220 .mu.m will work satisfactorily. However, depending upon the three-dimensional structure and intricacy of the matrix, other sizes may work equally well. In fact, any shape or structure that allow the stromal cells to stretch and continue to replicate and grow for lengthy time periods will work in accordance with the invention.

Detailed Description Text (29):
Different proportions of the various types of collagen deposited on the matrix can affect the growth of the later inoculated tissue-specific cells. For example, for optimal growth of hematopoietic cells, the matrix should preferably contain collagen types III, IV and I in an approximate ratio of 6:3:1 in the initial matrix. For three-dimensional skin culture systems, collagen types I and III are preferably deposited in the initial matrix. The proportions of collagen types deposited can be manipulated or enhanced by selecting fibroblasts which elaborate the appropriate collagen type. This can be accomplished using monoclonal antibodies of an appropriate isotype or subclass that is capable of activating complement, and which define particular collagen types. These antibodies and complement can be used to negatively select the fibroblasts which express the desired collagen type. Alternatively, the stroma used to inoculate the matrix can be a mixture of cells which synthesize the appropriate collagen types desired. The distribution and origins of the five types of collagen is shown in Table I.

Detailed Description Text (30):
Thus, depending upon the tissue to be cultured and the collagen types desired, the appropriate stromal cell(s) may be selected to inoculate the three-dimensional matrix.

Detailed Description Text (31):
During incubation of the three-dimensional stromal support, proliferating cells may be released from the matrix. These released cells may stick to the walls of the culture vessel where they may continue to proliferate and form a confluent monolayer. This should be prevented or minimized, for example, by removal of the released cells during feeding, or by transferring the three-dimensional stromal matrix to a new culture vessel. The presence of a confluent monolayer in the vessel will "shut down" the growth of cells in the three-dimensional matrix and/or culture. Removal of the confluent monolayer or transfer of the matrix to fresh media in a new vessel will restore proliferative activity of the three-dimensional culture system. Such removal or transfers should be done in any culture vessel which has a stromal monolayer exceeding 25% confluency. Alternatively, the culture system could be agitated to prevent the released cells from sticking, or instead of periodically feeding the cultures, the culture system could be set up so that fresh media continuously flows through the system. The flow rate could be adjusted to both maximize proliferation within the three-dimensional culture, and to wash out and remove cells released from the matrix, so that they will not stick to the walls of the vessel and grow to confluence. In any case, the released stromal cells can be collected and cryopreserved for future use.

Detailed Description Text (32):

5.2. Inoculation of Tissue-Specific Cells onto Three-Dimensional Stromal Matrix and Maintenance of Cultures

Detailed Description Text (33):
Once the three-dimensional stromal matrix has reached the appropriate degree of growth, the tissue-specific cells (parenchymal cells) which are desired to be cultured are inoculated onto the stromal matrix. A high concentration of cells in the inoculum will advantageously result in increased proliferation in culture much sooner than will low concentrations. The cells chosen for inoculation will depend upon the tissue to be cultured, which may include but is not limited to bone marrow, skin, liver, pancreas, kidney, neurological tissue, and adrenal gland, to name but a few.

Detailed Description Text (34):
For example, and not by way of limitation, a variety of epithelial cells can be cultured on the three-dimensional living stromal support. Examples of such epithelial cells include, but are not limited to, oral mucosa and gastrointestional (G.I.) tract cells. Such epithelial cells may be isolated by enzymatic treatment of the tissue according to methods known in the art, followed by expansion of these cells in culture and application of epithelial cells to the three-dimensional stromal support cell matrix (neo-submucosa). The presence of the submucosa provides growth factors and other proteins which promote normal division and differentiation of the oral mucosa cells and the cells of the G.I. tract lining. Using this methodology other epithelial cells can be grown successfully, including nasal epithelium, respiratory tract epithelium, vaginal epithelium, and corneal epithelium.

Detailed Description Text (35):
A variety of tumors may be grown on the three-dimensional living stromal support. Examples of such tumors include but are not limited to adenocarcinoma and malignant melanoma which may be derived from primary or metastatic sites. Such cultures may be established in a manner similar to other three-dimensional epithelial cultures. Briefly, stromal cells, derived from either the patient's tumor or normal tissue or from an allogeneic source, are established on the mesh. After reaching near-confluency the stromal cells are inoculated with tumor cells. The tumor cells will continue to divide rapidly and form a three-dimensional solid tumor. Tumor cells grown in such a three-dimensional support exhibit a morphology similar to the in vivo state and express and shed surface antigens in a manner similar to that of solid tumors; malignant cells grown in monolayers do not exhibit the same degree of similarity to in vivo tumor tissue. Such a physiological growth of tumor cells allows applications in the study and development of new chemotherapeutic agents, individualized chemotherapy regimens, and mechanisms of metastasis. In addition such tumor cultures may be useful in individualized immunotherapy. In this regard experimentation with hu 51CR release studies has indicated that Lak cells evoke a much more potent response against tumor cells grown in three-dimensions as compared to cells cultured in monolayer. Immune cells may be obtained from patients by traditional pheresis techniques and sensitized to the patient's own tumor cells grown in three-dimensional culture.

Detailed Description Text (37):
The parenchymal or tissue-specific cells used in the inoculum may be obtained from cell suspensions prepared by disaggregating the desired tissue using standard techniques described for obtaining stromal cells in Section 5.1 above. The entire cellular suspension itself could be used to inoculate the three-dimensional stromal support matrix. As a result, the regenerative cells contained within the homogenate will proliferate, mature, and differentiate properly on the matrix, whereas non-regenerative cells will not. Alternatively, particular cell types may be isolated from appropriate fractions of the cellular suspension using standard techniques described for fractionating stromal cells in Section 5.1 above. Where the "stem" cells or "reserve" cells can be readily isolated, these may be used to

preferentially inoculate the three-dimensional stromal support. For example, when culturing bone marrow, the three-dimensional stroma may be inoculated with bone marrow cells, either fresh or derived from a cryopreserved sample. When culturing skin, the three-dimensional stroma may be inoculated with melanocytes and keratinocytes. When culturing liver, the three-dimensional stroma may be inoculated with hepatocytes. When culturing pancreas, the three-dimensional stroma may be inoculated with pancreatic endocrine cells. For a review of methods which may be utilized to obtain parenchymal cells from various tissues, see, Freshney, Culture of Animal Cells. A Manual of Basic Technique, 2d Ed., A. R. Liss, Inc., New York, 1987, Ch. 20, pp. 257-288.

Detailed Description Text (41):
The three-dimensional culture system of the invention can be used in a variety of applications. These include but are not limited to transplantation or implantation of either the cultured cells obtained from the matrix, or the cultured matrix itself in vivo; screening cytotoxic compounds, allergens, growth/regulatory factors, pharmaceutical compounds, etc., in vitro; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; diagnosing and monitoring cancer in a patient; gene therapy; and the production of biologically active products, to name but a few.

Detailed Description Text (43):
Three-dimensional tissue culture implants may, according to the invention, be used to replace or augment existing tissue, to introduce new or altered tissue, to modify artificial prostheses, or to join together biological tissues or structures. For example, and not by way of limitation, specific embodiments of the invention would include (i) three-dimensional bone marrow culture implants used to replace bone marrow destroyed during chemotherapeutic treatment; (ii) three-dimensional liver tissue implants used to augment liver function in cirrhosis patients; (iii) genetically altered cells grown in three-dimensional culture (such as three-dimensional cultures of fibroblasts which express a recombinant gene encoding insulin); (iv) hip prostheses coated with three-dimensional cultures of cartilage; (v) dental prostheses joined to a three-dimensional culture of oral mucosa.

Detailed Description Text (44):
The three-dimensional cultures may be used in vitro to screen a wide variety of compounds, such as cytotoxic compounds, growth/regulatory factors, pharmaceutical agents, etc. To this end, the cultures are maintained in vitro and exposed to the compound to be tested. The activity of a cytotoxic compound can be measured by its ability to damage or kill cells in culture. This may readily be assessed by vital staining techniques. The effect of growth/regulatory factors may be assessed by analyzing the cellular content of the matrix, e.g., by total cell counts, and differential cell counts. This may be accomplished using standard cytological and/or histological techniques including the use of immunocytochemical techniques employing antibodies that define type-specific cellular antigens. The effect of various drugs on normal cells cultured in the three-dimensional system may be assessed. For example, drugs that increase red blood cell formation can be tested on the three-dimensional bone marrow cultures. Drugs that affect cholesterol metabolism, e.g., by lowering cholesterol production, could be tested on the three-dimensional liver system. Three-dimensional cultures of tumor cells may be used as model systems to test, for example, the efficacy of anti-tumor agents.

Detailed Description Text (46):
The three-dimensional cell cultures may also be used to aid in the diagnosis and treatment of malignancies and diseases. For example, a biopsy of any tissue (e.g. bone marrow, skin, liver, etc.) may be taken from a patient suspected of having a malignancy. If the biopsy cells are cultured in the three-dimensional system of the invention, malignant cells will be clonally expanded during proliferation of the culture. This will increase the chances of detecting a malignancy and, therefore, increase the accuracy of the diagnosis. This may be especially useful in diseases

such as AIDS where the infected population of cells is depleted in vivo. Moreover, the patient's culture could be used in vitro to screen cytotoxic and/or pharmaceutical compounds in order to identify those that are most efficacious; i.e. those that kill the malignant or diseased cells, yet spare the normal cells. These agents could then be used to therapeutically treat the patient.

Detailed Description Text (50):
The three-dimensional culture system of the invention may afford a vehicle for introducing genes and gene products in vivo for use in gene therapies. For example, using recombinant DNA techniques, a gene for which a patient is deficient could be placed under the control of a viral or tissue-specific promoter. The recombinant DNA construct containing the gene could be used to transform or transfect a host cell which is cloned and then clonally expanded in the three-dimensional culture system. The three-dimensional culture which expresses the active gene product, could be implanted into an individual who is deficient for that product.

Detailed Description Text (51):
The use of the three-dimensional culture in gene therapy has a number of advantages. Firstly, since the culture comprises eukaryotic cells, the gene product will be properly expressed and processed in culture to form an active product. Secondly, gene therapy techniques are useful only if the number of transfected cells can be substantially enhanced to be of clinical value, relevance, and utility; the three-dimensional cultures of the invention allow for expansion of the number of transfected cells and amplification (via cell division) of transfected cells.

Detailed Description Text (54):
In a further embodiment of the invention, three-dimensional cultures may be used to facilitate gene transduction. For example, and not by way of limitation, three-dimensional cultures of fibroblast stroma comprising a recombinant virus expression vector may be used to transfer the recombinant virus into cells brought into contact with the stromal matrix, thereby simulating viral transmission in vivo. The three-dimensional culture system is a more efficient way of accomplishing gene transduction than are current techniques for DNA tansfection.

Detailed Description Text (55):
In yet another embodiment of the invention, the three-dimensional culture system could be use in vitro to produce biological products in high yield. For example, a cell which naturally produces large quantities of a particular biological product (e.g., a growth factor, regulatory factor, peptide hormone, antibody, etc.), or a host cell genetically engineered to produce a foreign gene product, could be clonally expanded using the three-dimensional culture system in vitro. If the transformed cell excretes the gene product into the nutrient medium, the product may be readily isolated from the spent or conditioned medium using standard separation techniques (e.g., HPLC, column chromatography, electrophoretic techniques, to name but a few). A "bioreactor" could be devised which would take advantage of the continuous flow method for feeding the three-dimensional cultures in vitro. Essentially, as fresh media is passed through the three-dimensional culture, the gene product will be washed out of the culture along with the cells released from the culture. The gene product could be isolated (e.g., by HPLC column chromatography, electrophoresis, etc) from the outflow of spent or conditioned media.

Detailed Description Text (66):
6.2. Establishment of the Three-Dimensional Stromal Matrix

Detailed Description Text (68):
The cells are then plated onto the three-dimensional matrix. If high concentrations of stromal cells are used in the inoculum, the stromal support matrix will achieve the appropriate degree of subconfluency in shorter time periods. For example,

approximately 10.sup.6 to 10.sup.7 stromal cells per ml may be plated onto a three-dimensional matrix such as sterile nylon mesh (Tetko Corp. of New York, N.Y., USA) contained in a petri dish or other suitable chamber (e.g., Titer-Tek containers).

Detailed Description Text (70):
Suspended stromal cells growing in the three-dimensional matrix can be cryopreserved using the same technique as previously described for bone marrow cells. For cryopreservation of sub-confluent cells on the mesh, the nylon mesh may be rolled and inserted into a Nunc tube containing suitable medium such as RPMI 1640 supplemented with cryoprotectants such as dimethylsulfoxide and glycerol in final concentrations of about 5% and 15% respectively. Freezing of the stromal cells on the mesh can be accomplished at initial cooling rates of -1.degree. C./minute from +1.degree. C. to -40.degree. C. A cooling rate of -2.degree. to -3.degree. C./minute is optimum until the end stage temperature of -84.degree. C. is achieved. Approximately 20-25% of the stromal cells may detach from the nylon mesh during this process.

Detailed Description Text (72):
The primary rate limiting factor in the growth of marrow stromal cells is the relatively low mitotic index of the fibroblasts included among the marrow stromal cells. The growth of these cells and their deposition of extracellular matrix components may be enhanced by adding hydrocortisone hemisuccinate and/or self-regulating growth factors derived from the medium of cultured human fetal fibroblasts which have a high rate of cell division.

Detailed Description Text (74):
In one embodiment of the invention, growth enhancing fibroblasts that are synthesizing collagen and other extracellular matrix components are grown on the mesh until they reach subconfluency. A mixture of both hematopoietic and stromal bone marrow cells are then inoculated onto the subconfluent growth enhancing fibroblast meshwork.

Detailed Description Text (82):
Growth enhancing fibroblasts can be cryopreserved using the same techniques as previously described for stromal cells. Like the stromal cells, some of the growth enhancing fibroblasts will also detach from the mesh during freezing. This matrix, however, still contributes to the attachment of marrow stromal cells and therefore diminishes the time required for the establishment of a matrix conducive to hematopoietic cell growth.

Detailed Description Text (84):
Bone marrow cells are suspended in an appropriate nutrient medium (e.g., RPMI/1640 supplemented with FBS, HS, hydrocortisone, and appropriate antibiotics could be used) and inoculated onto the three-dimensional stromal support. These cells may either be fresh or derived from a formerly cryopreserved sample which has been rapidly thawed, for example, in an 80.degree. C. hot water bath. A suitable concentration of cells are inoculated onto subconfluent stromal cell meshworks. For example, 10.sup.6 to 10.sup.7 cells can be inoculated onto the three-dimensional stromal matrices in 25 mm.sup.2 plastic culture flasks and grown at about 33.degree. C. to 34.degree. C. and 5% CO.sub.2 in ambient air. The relative humidity of these cultures should be in excess of about 90%. After 3 days, the culture temperature should be raised to about 35.degree. C. to 37.degree. C.

Detailed Description Text (85):
In general, hematopoietic cells will grow in the natural pockets formed by the subconfluent stromal cells and the progenitor cells will remain in the adherent layer of cells. The adherent layer are those cells attached directly to the mesh or those connected indirectly by attachment to cells that are themselves attached directly to the mesh. Although hematopoietic colonization occurs rapidly, stromal seeding appears to be the rate limiting step for hematopoiesis, since the

hematopoietic cells from the inoculum seed mainly those areas where a stromal support matrix is present. Colonization occurs in the natural interstices formed by the partially developed stromal layers and is also seen on the outermost surface of the matrix. The surface colonies are somewhat smaller than those in the matrix and appear, at times, to be part of the non-adherent zone. Actually, they are loosely attached and remain after feeding. These cells, which are also found consistently in monolayer type LTBMC, have been termed the "pseudo-adherent layer" (Coulombel et al., 1983, Blood 62:291-297).

Detailed Description Text (99):
The three-dimensional bone marrow cultures of the present invention may be used for treating diseases or conditions which destroy healthy bone marrow cells or depress their functional ability. The process is effective especially in the treatment of hematological malignancies and other neoplasias which metastasize to the bone marrow. This aspect of the invention is also effective in treating patients whose bone marrow has been adversely affected by environmental factors, (e.g., radiation, toxins etc.), chemotherapy and/or radiation therapy necessitated by a disease which does not directly affect the bone marrow. In these cases, for example, bone marrow cells from a healthy patient can be removed, preserved, and then replicated and reinfused should the patient develop an illness which either destroys the bone marrow directly or whose treatment adversely affects the marrow.

Detailed Description Text (100):
The three-dimensional culture system of the present invention has several advantages to a patient in need of a bone marrow transplant. If the patient is receiving his or her own cells, this is called an autologous transplant; such a transplant has little likelihood of rejection. Autologous transplants eliminate a major cause of bone marrow transplant rejection, that is, the graft vs. host reaction. If the marrow contains malignant or diseased cells, small samples it can be more effectively purged when using the three-dimensional culture system of the invention. As previously explained, selective methods for purging malignant or diseased cells would work best in small volumes of bone marrow cells. The three-dimensional culture system described herein makes this feasible. Accordingly, a small sample obtained from the patient can be more efficiently purged using a selective method that kills malignant cells yet spares healthy cells. The remaining healthy cells can then be expanded considerably using the three-dimensional culture system of the invention. In addition, the process of the present invention allows more aggressive treatment of neoplastic disorders with chemotherapeutic agents and radiation. Presently, the extent of these treatments is often limited by bone marrow toxicity.

Detailed Description Text (102):
In a patient with cancer or other diseases, it is often efficacious to monitor the patient's condition by aspirating a portion of the patient's bone marrow and examining the sample. In this manner, a metastasis or recurrence may be detected before it is clinically obvious. Patients with other conditions that are detectable by examining bone marrow cells may also be monitored in this way.

Detailed Description Text (105):
The cytotoxicity to bone marrow of pharmaceuticals, anti-neoplastic agents, carcinogens, food additives, and other substances may be tested by utilizing the in vitro bone marrow replication system of the present invention.

Detailed Description Text (118):
While the use of fibroblasts alone is sufficient to form a three-dimensional stromal matrix that functions as a dermal equivalent, additional types of stromal cells may be used to inoculate the three-dimensional matrix. These include, but are not limited to endothelial cells, pericytes, macrophages, monocytes, lymphocytes, plasma cells, adipocytes, etc.

Detailed Description Text (120):
In order to culture full thickness skin, i.e., comprising both an epidermal and dermal layer, epidermal cells should be inoculated onto the dermal equivalent. To this end, melanocytes and keratinocytes may be inoculated simultaneously, or preferably, in sequence. For example, keratinocytes can be inoculated onto subconfluent melanocytes which were previously inoculated onto the stromal matrix.

Detailed Description Text (134):
Morphological characterization of the three-dimensional stroma indicate that the fibroblasts inoculated onto the matrix stretch across the openings, exhibit matrix deposition, and migrate into the interstices of the mesh. FIG. 1 illustrates the ability of the fibroblasts to arrange themselves into parallel layers between the naturally-secreted collagen bundles. These fibroblasts exhibit a rapid rate of cell division and protein secretion. Melanocytes will grow normally in the three-dimensional system in that they exhibit dendrite formation, remain pigmented and retain the ability to transfer pigment (see FIGS. 6 through 8).

Detailed Description Text (139):
To form the three-dimensional stromal matrix, it would be preferable to utilize skin fibroblasts obtained from the patient who is to receive the graft. Alternatively, fetal fibroblasts or a mixture of fetal fibroblasts and the patient's fibroblasts may be used. However, according to the invention, fibroblasts from autologous, allogeneic, or xenogeneic source may be used; Example Section 19 illustrates a specific embodiment of the invention in which human fibroblasts are cultured according to the invention, implanted and successfully grafted into pig. More importantly, however, the later inoculated epidermal cells may be advantageously derived from the patient in order to minimize the risk of rejection of the graft.

Detailed Description Text (140):
In an alternate embodiment of this aspect of the invention, the three-dimensional stromal support matrix which forms the neodermis can itself be engrafted onto the patient's wound. In this instance, the patient's own epidermal cells in the wound area will invade the stromal matrix and proliferate on the stromal matrix in vivo to form full thickness skin, i.e., both epidermal and dermal layers. Alternatively, epidermal cells may be seeded onto the neodermis, or sheets of epidermal cells may be applied. Where large wound areas are to be covered, it may be preferred to engraft the complete three-dimensional skin culture, or to use combinations of both neodermis and full-thickness skin cultures. For example, neodermis could be engrafted at the edges of the wound, and full thickness cultures in central areas of the wound, to enhance growth and healing and minimize scar formation.

Detailed Description Text (143):
The three-dimensional skin culture could be used as a substrate to test the cytotoxicity of compounds and other substances. For example, for use in cytotoxicity assays, human cells could be grown onto meshes which could be cut into 6 mm disks, places into 96-well flat bottom tissue culture microtest plates, and fed with appropriate medium. The test substance could then be added to each sample. The test substance could be advantageously applied by limiting dilution technique, in which case, a range of concentrations of the toxic substance can be tested. Each tissue type may be represented by three rows of meshes in order to provide data in triplicate. A properly controlled assay could be run as follows: mesh alone; mesh inoculated with fibroblasts; mesh inoculated with fibroblasts and keratinocytes; mesh with fibroblasts and melanocytes; and mesh inoculated with fibroblasts, melanocytes and keratinocytes. Chemical agents can be added to each of these substrates and incubated, e.g. for 24 hours. The cytotoxic effect of such substances can be evaluated in a number of ways. For example, a convenient method, the well known neutral red assay, could be adapted for use in this system. To this end, after removal of the medium, each well may be rinsed before adding a 0.4% aqueous stock solution of neutral red dye. After various time intervals the dye is

removed and cells are rapidly washed with 4.0% formaldehyde, 1.0% CaCl.sub.2. After about 20 minutes, the amount of dye present in each tissue sample can be measured by reading absorbance with a Dynatech microplate reader equipped with a 540 nm filter. The amount of vital dye absorbed is directly proportional to the number of viable cells present in each well. The readings can be averaged and the results expressed as absorbance observed over baseline levels in control cultures.

Detailed Description Text (150):
Liver cultures maintained in this fashion may be utilized for a variety of purposes including cytotocity testing, screening drugs, etc. In one embodiment, three-dimensional liver cultures could be used to screen for carcinogens and mutagens in vitro. More particularly, it is well known that a number of compounds fail to act as mutagens in test organisms such as bacteria or fungi, yet cause tumors in experimental animals such as mice. This is due to metabolic activation; i.e., some chemicals are metabolically altered by enzymes in the liver (the P450 oxidase system and hydroxylation systems) or other tissues, creating new compounds that are both mutagenic and carcinogenic. In order to identify such carcinogens, Ames and his co-workers devised a screening assay which involves incubating the chemical compound with liver extracts prior to exposure of the test organism to the metabolic product (Ames et al., 1975, Mut. Res. 31:347-364). While a more sophisticated approach, the Ames assay still lacks sensitivity. By contrast, the three-dimensional liver cultures can be utilized both as the metabolic converters and the "test organism" to determine the mutagenicity or carcinogenicity of the substance being tested.

Detailed Description Text (152):
According to the invention, a three-dimensional tissue culture model system for the blood-brain barrier may be produced. Briefly, this three-dimensional culture recreates the endothelial cell barrier which separates the central nervous system from the bloodstream by first growing endothelial cells derived from small blood vessels of the brain to confluence in a three-dimensional mesh. First astrocytes, and then neurons, are applied to the confluent stromal matrix formed by endothelial cells such that the endothelial cells form a barrier between one surface of the culture, above, and the neurons, below. A substance applied to the endothelial cell surface must penetrate through the endothelial cell layer to reach the neurons beneath.

Detailed Description Text (155):
Astrocytes may then be inoculated onto confluent endothelial cell three-dimensional stromal matrices, cultured for a period of about 5 days and then further inoculated with neuronal cells.

Detailed Description Text (161):
The results indicate that the three-dimensional culture system supports the expression of several hematologic lineages as evidenced by the differential counts of the non-adherent and adherent zones of the human, macaque and rat cells. Cytofluorographic analysis of the cells attached to the three-dimensional stroma, i.e., the adherent zone, revealed the presence of early and late myeloid precursors, mature granulocytes, B and T lymphocytes, megakaryocytes/platelets, and monocytes/macrophages. Although the number of progenitor cells located in the matrix was variable, this may have resulted from the random populations of stromal cells used to form the support matrix.

Detailed Description Text (171):
11.2. Establishment of the Three-Dimensional Stromal Matrix

Detailed Description Text (179):
11.3. Inoculation of Three-Dimensional Stromal Matrix with Hematopoietic Cells and Establishment of Culture

Detailed Description Text (197):
Human and monkey LTBMC can be established on a stratum of fetal human fibroblasts but this matrix will not support the growth of rat LTBMC. The fetal fibroblast cells reach a stage of subconfluence which will allow the subsequent inoculation of marrow cells much sooner than narrow stroma. When macaque bone marrow is grown on a bed of fetal fibroblasts, the phenotypic profile of the adherent zone shows that more cells react with the Plt-1 antibody than in the other cultures we studied but the other hematologic lineages are represented also (Table V). It is not known to what extent this finding reflects cross-reactivity of the antibody or a shift in the cell population of the adherent zone mediated by the fetal cells.

Detailed Description Text (202):
The subsections below describe the three-dimensional culture system of the invention for culturing skin in vitro. Briefly, cultures of fibroblasts were established on nylon mesh which had been previously sterilized. Within 6-9 days of incubation, adherent fibroblasts began to grow into the meshwork openings and deposited parallel bundles of collagen. Indirect immunofluorescence using monoclonal antibodies showed predominantly type I collagen with some type III as well. After 7 days, co-cultures of human melanocytes and kertinocytes were plated onto the fibroblast meshwork. No TPA or cholera toxin was added since trophic factors are produced by the subconfluent fibroblasts of the adherent layer. Electron microscopic studies revealed skin cells with normal morphological characteristics and cell-cell attachments.

Detailed Description Text (206):
Melanocytes were isolated according to the method of Eisinger and Marko (1982, Proc. Natl. Acad. Sci. USA 79:2018-2022). Briefly, skin samples were incubated in trypsin for 4-6 hours, allowing separation of the epidermal and dermal layers. Epidermal cells were suspended in media and plated into 25 cm.sup.2 Falcon tissue culture flasks. Melanocytes were separated from keratinocytes by preferential attachment qualities. Isolated melanocytes were plated onto the fibroblast-coated nylon mesh and allowed to grow for 3 days prior to the addition of keratinocytes. Melanocytes grow normally in this system in that they exhibit dendrite formation, remain pigmented, and retain the ability to transfer pigment to keratinocytes. FIG. 6 depicts the appearance of melanocytes after 3 days in the three-dimensional culture system. Isolated keratinocytes were plated onto the melanocytes after 3-4 days. This "tissue" grows rapidly and is maintained in RPMI 1640, 10% FBS, and the appropriate antibiotics. Since natural growth factors are secreted by the dermal elements, no addition of exogenous factors (e.g., TPA, cholera toxins, etc., as described by Sengel, 1983, in Biochemistry and Physiology of Skin, Vol.1, pp.102-131, Oxford Univ. Press, N.Y.; and Eisinger et al., 1988, Proc. Natl. Acad. Sci. USA 3085:1937-1941), is necessary.

Detailed Description Text (224):
13.1.4. Preparation of Three-Dimensional Stromal Matrix

Detailed Description Text (227):
After inoculation of liver parenchymal cells onto the three-dimensional stromal matrix, cultures were maintained in DMEM complete medium at 37.degree. C. and 5% CO.sub.2 in a humidified atmosphere and were fed with fresh medium every 3 days.

Detailed Description Text (233):
Samples of oral mucosal tissue were obtained from orthodontic surgical specimens. Tissue was washed three times with fresh MEM containing antibiotics (2 ml of antibiotic antimycotic solution, from GIBCO, Cat. #600-5240 AG; and 0.01 ml of gentamycin solution from GIBCO, Cat. #600-5710 AD per 100 cc MEM), cut into small pieces, then washed with 0.02% EDTA (w/v). 0.25% trypsin (in PBS without Ca++ or Mg++ was added; after a few seconds, the tissue pieces were removed and placed in fresh 0.25% trypsin (in PBS without Ca++ or Mg++) and refrigerated at 4.degree. C. overnight. Tissues were then removed and placed in fresh trypsin solution, and

gently aggitated until cells appeared to form a single-cell suspension. The single-cell suspension was then diluted in MEM containing 10% heat-inactivated fetal bovine serum and centifuged at 1400 g for 7 minutes. The supernatant was decanted and the pellet containing mucosal epithelial cells was placed into seeding medium. Medium consisted of DMEM with 2% Ultrosen G, 1.times.L-glutamine, 1.times.nonessential amino acids, penicillin and streptomycin. The cells were then seeded onto a three-dimensional stromal matrix (see infra).

Detailed Description Text (234):
14.1.2. Preparation of the Three-Dimensional Stromal Matrix

Detailed Description Text (235):
The three-dimensional stromal matrix used in mucosal epithelium cultures was generated using oral fibroblasts and 8 mm.times.45 mm pieces of nylon filtration screen (#3-210/36, Tetko Inc., N.Y.) as described above for three-dimensional liver cultures in Section 13.1.4).

Detailed Description Text (237):
After inoculation of mucosal epithelial cells onto the three-dimensional stromal matrix, cultures were maintained in DMEM complete medium at 37.degree. and 5% CO.sub.2 in a humidified atmosphere and were fed with fresh medium every 3 days.

Detailed Description Text (244):
15.1.2. Preparation of the Three-Dimensional Stromal Matrix

Detailed Description Text (245):
The three-dimensional stromal matrix used in pancreatic tissue cultures was generated using adult rat pancreatic fibroblasts and 8 mm.times.45 mm pieces of nylon filtration screen (#3-210/36, Tetko, Inc., N.Y.) as described above for three-dimensional liver cultures in Section 13.1.4.

Detailed Description Text (247):
After inoculation of pancreatic acinar cells onto the three-dimensional stromal matrix, cultures were maintained in DMEM complete medium at 37.degree. C. and 5% CO.sub.2 in a humidified atmosphere and were fed with fresh medium every 3 days.

Detailed Description Text (264):
Neurons and astrocytes were prepared from the cerebellum of fetal rats, and separated by differential adherence. Astrocytes were grown on the confluent endothelial cell three-dimensional stromal matrix, and, subsequently, neuronal cells were added to the three-dimensional tissue culture.

Detailed Description Text (269):
Adenocarcinoma cells were separated from stromal cells by mincing tumor cells in HBSS, incubating the cells in 0.27% trypsin for 24 hours at 37.degree. C. and further incubating suspended cells in DMEM complete medium on a plastic petri dish for 12 hours at 37.degree. C. Stromal cells selectively adhered to the plastic dishes.

Detailed Description Text (270):
17.1.2. Preparation of the Three-Dimensional Stromal Matrix

Detailed Description Text (271):
The three-dimensional stromal matrix used in adenocarcinoma tissue cultures was generated using stromal cells derived from the tumor (see Section 17.1.1., supra) and 8 mm.times.45 mm pieces of nylon filtration screen (#3-210/36, Tetko, Inc., N.Y), as described above for three-dimensional liver cultures in Section 13.1.4.

Detailed Description Text (273):
After inoculation of adenocarcinoma cells onto the three-dimensional tumor stromal

matrix, cultures were maintained in DMEM complete medium with high glucose, 15% FBC and 0.03% glutamine at 37.degree. C. and 5% CO.sub.2 in a humidified atmosphere and were fed with fresh medium every 3 days.

Detailed Description Text (275):
FIG. 16 is a photomicrograph of a three-dimensional adenocarcinoma tissue culture. Adenocarcinoma cells showed a characteristic piling and orientation into a three-dimensional tumor-like structure. Cells retained their epithelial-like appearance.

Detailed Description Text (280):
18.1.2. Exposure of Three-Dimensional Bone Marrow Cultures to Cytotoxic Agents

Detailed Description Text (296):
FIGS. 17 and 18 show the results of three-dimensional bone marrow culture cytotoxicity assays, using adriamycin and cis-platinum, respectively, as test agents. Note that, in each case, the three-dimensional culture systems show a dose-related response to test agent. Significantly, with either adriamycin or cis-platinum, the TD.sub.50 for bone marrow three-dimensional cultures was different from the TD.sub.50 determined using conventional fibroblast monolayer cultures. Importantly, these results indicate that monolayer cultures may not be accurate measures for cytotoxicity; perhaps because the cells are growing in an extremely unnatural environment, monolayer cell cultures may be more sensitive to toxic agents. It is crucial to be able to determine the actual toxicity of a test substance; for example, in chemotherapy, it may be important to administer the highest dose tolerable in order to effectively eliminate malignant cells. Underestimating the highest tolerated dose may result in administering a less effective amount of anti-tumor agent. By providing three-dimensional tissue cultures not only of bone marrow and other normal tissues, but tumor tissues as well, the present invention enables the in vitro determination of the optimal dose of chemotherapeutic agent.

Other Reference Publication (1):
Leighton, J., 1951, J. Natl. Cancer Inst. 12:545-561.

Other Reference Publication (5):
Leighton, J. et al., 1968, Cancer Res. 28:286-296.

Other Reference Publication (20):
Yang, J. et al., 1981, Cancer Res. 41:1021-1027.

Other Reference Publication (69):
Sirica, A., et al., 1980, Cancer Res. 40:3259-3267.

Other Reference Publication (76):
Anderson et al., 1987, "Human Gene Therapy Preclinical Data Document", pp. 1-99, submitted to the NIH RAC Human Gene Therapy Subcommittee on Apr. 24, 1987; received by Stanford University Library in Jun., 1987; publicly reviewed by the Subcommittee on Dec. 7, 1987.

Other Reference Publication (79):
Culver et al., 1990, "Retroviral-Mediated Gene Transfer into Cultured Lymphoid Cells as a Vehicle for Gene Therapy", J. Cell Biochem. Suppl. 12B:171.

Other Reference Publication (81):
Anson et al., 1987, "Towards Gene Therapy for Hemophilia B", Mol. Biol. Med. 4:11-20.

Other Reference Publication (82):
St. Louis and Verma, 1988, "An Alternative Approach to Somatic Cell Gene Therapy", Proc. Natl. Acad. Sci. USA 85:3150-3154.

Other Reference Publication (83):
Rosenberg et al., 1988, "Grafting Genetically Modified Cells to the Damaged Brain: Restorative Effects of NGF Expression", Science 242:1575-1578.

Other Reference Publication (89):
Anderson, 1984, "Prospects for Human Gene Therapy", Science 226:401-409.

Other Reference Publication (90):
Cline, 1985, "Perspectives for Gene Therapy: Inserting New Genetic Information into Mammalian Cells by Physical Techniques and Viral Vectors", Pharmac. Ther. 29:69-92.

Other Reference Publication (91):
Cline, 1987, "Gene Therapy: Current Status", Am J. Med. 83:291-297.

Other Reference Publication (92):
Friedman, 1989, "Progress Toward Human Gene Therapy", Science 244:1275-1281.

Other Reference Publication (93):
Miller, 1992, "Human Gene Therapy Comes of Age", Nature 357:455-460.

CLAIMS:

11. A method for culturing a genetically engineered dermis comprising culturing in vitro stromal cells transfected with an exogenous gene under the control of an expression element comprising culturing the transfected stromal cells inoculated on a three-dimensional framework so that the transfected stromal cells and connective tissue proteins naturally secreted by the stromal cells attach to and substantially envelope the framework composed of a biocompatible, non-living material formed into a three-dimensional structure having interstitial spaces bridged by the stromal cells.

31. The method for culturing genetically engineered cells according to claim 21, in which the parenchymal cells are hematopoietic cells.

32. The method for culturing genetically engineered cells according to claim 21, in which the parenchymal cells are melanocytes and keratinocytes.

33. The method for culturing genetically engineered culture according to claim 21, in which the transfected parenchymal cells are liver parenchymal cells.

34. The method for culturing genetically engineered culture according to claim 21, in which the transfected parenchymal cells are pancreatic acinar cells.

35. The method for culturing genetically engineered culture according to claim 21, in which the transfected parenchymal cells are kidney cells.

36. The method for culturing genetically engineered culture according to claim 21, in which the transfected parenchymal cells are mucosal epithelium cells.

39. A method for producing a biological product in a genetically engineered living stromal tissue comprising:

(a) culturing stromal cells transfected with an exogenous gene under the control of an expression element so that the exogenous gene product is expressed in culture, and in which the transfected stromal cells and connective tissue proteins naturally secreted by the transfected stromal cells are attached to and substantially envelope a framework composed of a biocompatible, non-living material formed into a three-dimensional structure having interstitial spaces bridged by the stromal

cells; and

(b) isolating the exogenous gene product from the culture.

40. A method for producing a biological product in a three-dimensional cell culture comprising:

(a) culturing parenchymal cells inoculated onto a genetically engineered living stromal tissue, comprising stromal cells transfected with an exogenous gene under the control of an expression element, so that the exogenous gene product is expressed in culture, and in which the transfected stromal cells and connective tissue proteins naturally secreted by the transfected stromal cells attached to and substantially enveloping a framework composed of a biocompatible, non-living material formed into a three-dimensional structure having interstitial spaces bridged by the transfected stromal cells; and

(b) isolating the exogenous gene product from the culture.

49. A method for producing a biological product in a genetically engineered dermis comprising:

(a) culturing stromal cells transfected with an exogenous gene under the control of an expression element so that the exogenous gene product is expressed in culture, and in which the transfected stromal cells and connective tissue proteins naturally secreted by the transfected stromal cells are attached to and substantially envelope a framework composed of a biocompatible, non-living material formed into a three-dimensional structure having interstitial spaces bridged by the stromal cells; and

(b) isolating the exogenous gene product from the culture.

50. A method for producing a biological product in a genetically engineered three-dimensional cell culture, comprising:

(a) culturing transfected parenchymal cells inoculated onto a living stromal tissue prepared in vitro comprising stromal cells and connective tissue proteins naturally secreted by the stromal cells attached to and substantially enveloping a framework composed of a biocompatible, non-living material formed into a three-dimensional structure having interstitial spaces bridged by the stromal cells, so that the inoculated cells proliferate, in which the transfected parenchymal cells contain an exogenous gene under the control of an expression element so that the exogenous gene product is expressed in the culture; and

(b) isolating the exogenous gene product from the culture.

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L28: Entry 484 of 570 File: USPT Jan 12, 1999

US-PAT-NO: 5858721
DOCUMENT-IDENTIFIER: US 5858721 A

TITLE: Three-dimensional cell and tissue culture system

DATE-ISSUED: January 12, 1999

INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/69.1; 435/1.1, 435/320.1, 435/325, 435/326, 435/347, 435/363, 435/365.1, 435/366, 435/367, 435/368, 435/369, 435/370, 435/371, 435/372, 435/373, 435/396, 435/397, 435/398, 435/399, 435/402, 435/69.2, 435/69.3, 435/69.4, 435/69.5, 435/69.6, 435/70.1

L28: Entry 489 of 570 File: USPT Dec 15, 1998

DOCUMENT-IDENTIFIER: US 5849588 A
TITLE: Methods of use of a three-dimensional liver cell and tissue culture system

Abstract Text (2):
The liver cultures have a variety of applications ranging from transplantation or implantation in vivo, to screening cytotoxic compounds and pharmaceutical compounds in vitro, to the production of biologically active molecules in "bioreactors" and to the construction of extracorporeal liver assist device.

Brief Summary Text (3):
The liver cultures have a variety of applications ranging from transplantation or implantation in vivo, to screening cytotoxic compounds and pharmaceutical compounds in vitro, to the production of biologically active molecules in "bioreactors" and to the construction of extracorporeal liver assist device.

Brief Summary Text (7):
In an attempt to study the diverse liver functions and the cell types responsible therefor, in vitro cultures of liver cells have been prepared from humans as well as from experimental animals. Primary cultures of rat hepatocytes have been used extensively to study the effects of potential toxins on enzyme leakage, metabolism, and cellular membranes (Grisham, 1979, Int. Rev. Exp. Pathol. 20:123-210; Acosta and Mitchell, 1981, Biochem. Pharmacol. 30:3225-3230). However, such culture systems have a number of drawbacks, and none have provided for the proliferation of liver PC.

Brief Summary Text (10):
Substantial improvements in this regard were produced by culturing PC with various types of non-parenchymal stromal or littoral hepatic cells or non-hepatic stromal cells. Both human and rat hepatocytes which were co-cultured with liver endothelial cells of the same species maintained specific functions for weeks in culture, although they did not undergo a significant expansion in numbers (Guguen-Guilluozo, et al., 1983, Exp. Cell Res. 143:47-54; Begue et al., 1983, Biochem. Pharmacol. 32:1643-1646). Rat hepatocytes which were co-cultured with human fibroblasts (Kuri-Harcuch and Mendoza-Figueroa, 1989, Differentiation 41:148-157) and endothelial cells (Begue et al., 1983, Biochem. Pharmacol. 32:1643-1646) were reported to sustain cytochrome P-450 activity for more than 10 days. Thus, these mixed hepatocyte co-culture systems may provide microenvironments similar to those in vivo by optimizing cell-cell interactions. In addition, various PC functions may be regulated and/or optimized by other hepatic cells. For example, Kupffer cell secretory products have been reported to modulate PC cytochrome P-450 enzyme activity (Peterson and Renton, 1984, J. Pharmacol. Exp. Ther. 229:299-304). The attachment of PC to fibroblasts is evidently contingent upon the secretion of specialized extracellular matrix substances by Kupffer cells (Michalopoulos et al., 1979, In Vitro 15:769-806). Hepatic endothelial cells also may produce important components of the extracellular matrix (Guguen-Guilluozo, et al., 1983, Exp. Cell Res. 143:47-54), and adipocytes may provide the requisite raw materials for the renewal of cell membranes in metabolically-active hepatocytes.

Brief Summary Text (18):
The invention is described by way of examples in which adult rat PC are cultured

for long-terms in the presence of stromal cells which are grown on a three-dimensional support. Cells derived from the liver PC: stroma co-cultures exhibit a structural and functional heterogeneity as do liver cells in vivo. Proliferation of PC occurs in vitro and appears to be contingent upon the geometry of the culture framework; and, when established on biodegradable framework, these liver PC: stromal cell co-cultures are capable of regenerating a liver-like architecture at ectopic sites and retain their ability to synthesize liver-specific proteins. This. liver cell and tissue culture system may have applications as a substrate for hepatotoxicity testing or in an extracorporeal liver assist device and, when grown on a biogradable polymer framework, to be implanted into subjects with inborn errors of metabolism. Furthermore, genetically engineered liver cells maintain the expression of their exogenous gene long term when grown in the culture system of the present invention.

Drawing Description Text (15):
FIG. 8A Photomicrographs of co-cultures of liver PC and stromal cells on PGA felt 30 days after grafting into Long-Evans rats. H and E staining. Low power view of a subcutaneous graft showing a focus of hepatic tissue (H) contiguous to connective tissue (C) in the process of reorganization. A tract of residual, partially hydrolyzed PGA polymers (p) is present. Arrows identify putative biliary structures associated with the regenerating hepatic tissue. 100.times..

Detailed Description Text (4):
In accordance with the invention, liver PC are inoculated and cultured on a pre-established three-dimensional stromal tissue. The stromal tissue comprises stromal cells grown on a three-dimensional matrix or framework. The stromal cells comprise fibroblasts with or without additional cells and/or elements described more fully herein. The fibroblasts and other cells and/or elements that comprise the stroma may be fetal or adult in origin, and may be derived from convenient sources such as skin, liver, pancreas, etc. Such tissues and/or organs can be obtained by appropriate biopsy or upon autopsy. In fact, cadaver organs may be used to provide a generous supply of stromal cells and elements.

Detailed Description Text (5):
Neonatal fibroblasts may support the growth of many different cells and tissues in the three-dimensional culture system, and, therefore, can be inoculated onto the matrix to form a "generic" stromal tissue for culturing any of a variety of cells and tissues, including liver PC. However, in certain instances, it may be preferable to use a "specific" rather than "generic" stromal tissue, in which case stromal cells and elements can be obtained from a liver tissue. For example, where the three-dimensional culture is to be used for purposes of transplantation or implantation in vivo, it may be preferable to obtain the stromal cells and elements from the individual who is to receive the transplant or implant. This approach might be especially advantageous where immunological rejection of the transplant and/or graft versus host disease is likely. Moreover, fibroblasts and other stromal cells and/or elements may be derived from the same type of tissue to be cultured in the three-dimensional system. This might be advantageous when culturing liver tissues in which specialized stromal cells may play particular structural/functional roles; e.g., Kupffer cells of liver.

Detailed Description Text (17):
5.1. ESTABLISHMENT OF THREE-DIMENSIONAL STROMAL MATRIX

Detailed Description Text (18):
The three-dimensional support framework may be of any material and/or shape that: (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. A number of different materials may be used to form the framework, including but not limited to: nylon (polyamides), dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (e.g., polyvinylchloride), polycarbonate (PVC),

polytetrafluorethylene (PTFE; teflon), thermanox (TPX), nitrocellulose, cotton, polyglycolic acid (PGA), cat gut sutures, cellulose, gelatin, dextran, etc. Any of these materials may be woven into a mesh, for example, to form the three-dimensional framework. Certain materials, such as nylon, polystyrene, etc., are poor substrates for cellular attachment. When these materials are used as the three-dimensional support framework, it is advisable to pre-treat the matrix prior to inoculation of stromal cells in order to enhance the attachment of stromal cells to the framework. For example, prior to inoculation with stromal cells, nylon screens could be treated with 0.1M acetic acid, and incubated in polylysine, FBS, and/or collagen to coat the nylon. Polystyrene could be similarly treated using sulfuric acid.

Detailed Description Text (25):
Again, where the cultured cells are to be used for transplantation or implantation in vivo it is preferable to obtain the stromal cells from the patient's own tissues. The growth of cells in the presence of the three-dimensional stromal support framework may be further enhanced by adding to the framework, or coating it with proteins (e.g., collagens, elastin fibers, reticular fibers) glycoproteins, glycosaminoglycans (e.g., heparan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, etc.), a cellular matrix, and/or other materials.

Detailed Description Text (29):
Different proportions of the various types of collagen deposited on the matrix can affect the growth of the later inoculated PC. The proportions of ECM proteins deposited can be manipulated or enhanced by selecting fibroblasts which elaborate the appropriate collagen type. This can be accomplished using monoclonal antibodies of an appropriate isotype or subclass that is capable of activating complement, and which define particular collagen types. These antibodies and complement can be used to negatively select the fibroblasts which express the desired collagen type. Alternatively, the stroma used to inoculate the framework can be a mixture of cells which synthesize the appropriate collagen types desired. The distribution and origins of the five types of collagen is shown in Table I.

Detailed Description Text (31):
During incubation of the three-dimensional stromal support, proliferating cells may be released from the framework. These released cells may stick to the walls of the culture vessel where they may continue to proliferate and form a confluent monolayer. This should be prevented or minimized, for example, by removal of the released cells during feeding, or by transferring, the three-dimensional stromal tissue to a new culture vessel. The presence of a confluent monolayer in the vessel may "shut down" the growth of cells in the three-dimensional culture. Removal of the confluent monolayer or transfer of the stromal tissue to fresh media in a new vessel will restore proliferative activity of the three-dimensional culture system. Such removal or transfers should be done in any culture vessel which has a stromal monolayer exceeding 25% confluency. Alternatively, the culture system could be agitated to prevent the released cells from sticking, or instead of periodically feeding the cultures, the culture system could be set up so that fresh media continuously flows through the system. The flow rate could be adjusted to both maximize proliferation within the three-dimensional culture, and to wash out and remove cells released from the matrix, so that they will not stick to the walls of the vessel and grow to confluence. In any case, the released stromal cells can be collected and crypreserved for future use.

Detailed Description Text (36):
Furthermore, liver cultures are often difficult to evaluate. Several reasons for this include: mitoses in co-cultures of hepatic cells have been ascribed to non-parenchymal elements (Guguen-Guilluozo et al., 1983, Exp. Cell Res. 143:47-54), non-parenchymal liver cells have been reported to express functions similar to hepatocytes such as albumin synthesis (Hayner et al. 1988., Cancer Res. 48:368-

378), and hepatocyte phenotypes appear to change under some culture conditions even though PC and hepatic stroma remain morphologically distinct (Grisham, 1980, Ann. N.Y. Acad. Sci. 349:128-137).

Detailed Description Text (41):
The characteristics of cell growth on three-dimensional frameworks are intrinsically different from that on flat-bottomed plastic flasks. For example, the cell matrix deposition is enhanced, but proliferation rate is lower when stromal cells are cultured on nylon screens as compared to plastic flasks. This three-dimensional framework may also enhance the opportunity for normal cell-cell interactions and orientation, thereby permitting the various subpopulations of cells to act deterministically to form a tissue-like construct. In this regard, the presence of inhibitors in serum has been hypothesized as a potential reason why PC fail to proliferate in culture (Barnes and Sato, 1980, Cell 22:649-655) and serum factors have been reported to contribute to the appearance of the large, bizarre, and putatively de-differentiated masses of PC in monolayer cultures (Grisham, 1980, Ann. N.Y. Acad. Sci. 349:128-137; Hayner et al., 1988, Cancer Res. 48:368-378). These cells do not arise in nylon screen co-cultures, regardless of the length of the culture or the presence of serum in the medium. In addition, the serum conditioning that has been reported to adversely affect cP450 function in hepatocytes cultured on plastic flasks does not influence cP450 in the present model. The liver cultures disclosed herein are fed with SFM in order to eliminate non-specific protein binding for ELISA assays but this medium lacks sufficient nutrients to maintain the cells for long term. The most obvious deficiency of this SFM is its inability to support stromal cells, which begin to die after .about.48 h, an effect which is ameliorated but not completely abrogated by hydrocortisone supplementation.

Detailed Description Text (46):
The three-dimensional liver culture system of the invention can be used in a variety of applications. These include but are not-limited to transplantation or implantation of the cultured cells in vivo; screening cytotoxic compounds, carcinogens, mutagens growth/regulatory factors, pharmaceutical compounds, etc., in vitro; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; diagnosing and monitoring cancer in a patient; gene therapy; and the production of biologically active products, to name but a few.

Detailed Description Text (48):
The three-dimensional liver cultures may be used In vitro to screen a wide variety of compounds, such as cytotoxic compounds, growth/regulatory factors, pharmaceutical agents, etc. To this end, the cultures are maintained in vitro and exposed to the compound to be tested. The activity of a cytotoxic compound can be measured by its ability to damage or kill cells in culture. This may readily be assessed by vital staining techniques. The effect of growth/regulatory factors may be assessed by analyzing the cellular content of the matrix, e.g., by total cell counts, and differential cell counts. This may be accomplished using standard cytological and/or histological techniques including the use of immunocytochemical techniques employing antibodies that define type-specific cellular antigens. The effect of various drugs on normal cells cultured in the three-dimensional system may be assessed. For example, drugs that affect cholesterol metabolism, by lowering cholesterol production, could be tested on the three-dimensional liver system.

Detailed Description Text (49):
It is well known that a number of compounds fail to act as mutagens in test organisms such as bacteria or fungi, yet cause tumors in experimental animals such as mice. This is due to metabolic activation; i.e., some chemicals are metabolically altered by enzymes in the liver (the P450 oxidase system and hydroxylation systems) or other tissues, creating new compounds that are both mutagenic and carcinogenic. In order to identify such carcinogens, Ames and his co-

workers devised a screening assay which involves incubating the chemical compound with liver extracts prior to exposure of the test organism to the metabolic product (Ames et al., 1975, Mut. Res. 31:347-364). While a more sophisticated approach, the Ames assay still lacks sensitivity. By contrast, the three-dimensional liver cultures can be utilized both as the metabolic converters and the "test organism" to determine the mutagenicity or carcinogenicity of the substance being tested.

Detailed Description Text (50):
The three-dimensional cell cultures may also be used to aid in the diagnosis and treatment of malignancies and diseases. For example, a biopsy of liver tissue may be taken from a patient suspected of having a malignancy. If the biopsy cells are cultured in the three-dimensional system of the invention, malignant cells will be clonally expanded during proliferation of the culture. This will increase the chances of detecting a malignancy and, therefore, increase the accuracy of the diagnosis. Hepatitis virus-infected liver cells may be grown in the culture system of the invention. Moreover, the patient's culture could be used in vitro to screen cytotoxic and/or pharmaceutical compounds in order to identify those that are most efficacious; i.e. those that kill the malignant or diseased cells, yet spare the normal cells. These agents could then be used to therapeutically treat the patient.

Detailed Description Text (51):
The three-dimensional culture system of the invention may afford a vehicle for introducing genes and gene products in vivo for use in gene therapies. For example, using recombinant DNA techniques, a gene for which a patient is deficient could be placed under the control of a viral or tissue-specific promoter. The recombinant DNA construct containing the gene could be used to transform or transfect a host cell which is cloned and then clonally expanded in the three-dimensional culture system. The three-dimensional culture which expresses the active gene product, could be implanted into an individual who is deficient for that product.

Detailed Description Text (52):
The use of the three-dimensional culture in gene therapy has a number of advantages. Firstly, since the culture comprises eukaryotic cells, the gene product will be properly expressed and processed in culture to form an active product. Secondly, gene therapy techniques are useful only if the number of transfected cells can be substantially enhanced to be of clinical value, relevance, and utility; the three-dimensional cultures of the invention allow for expansion of the number of transfected cells and amplification (via cell division) of transfected cells.

Detailed Description Text (55):
In yet another embodiment of the invention, the three-dimensional culture system could be used in vitro to produce biological products in high yield. For example, a cell which naturally produces large quantities of a particular biological product (e.g., a growth factor, regulatory factor, peptide hormone, antibody, etc.), or a host cell genetically engineered to produce a foreign gene product, could be clonally expanded using the three-dimensional culture system in vitro. If the transformed cell excretes the gene product into the nutrient medium, the product may be readily isolated from the spent or conditioned medium using standard separation techniques (e.g., HPLC, column chromatography, electrophoretic techniques, to name but a few). A "bioreactor" could be devised which would take advantage of the continuous flow method for feeding the three-dimensional cultures in vitro. Essentially, as fresh media is passed through the three-dimensional culture, the gene product will be washed out of the culture along with the cells released from the culture. The gene product could be isolated (e.g., by HPLC column chromatography, electrophoresis, etc.) from the outflow of spent or conditioned media.

Detailed Description Text (70):

(b) Cytochrome P-450 (cP450) assay. Cells were analyzed for evidence to cP450 enzyme activity by quantifying incremental fluorescein fluorescence in cells accumulating ethoxyfluorescein ethyl ester (EFEE) (Miller, 1983, Anal. Chem. 133:46-57; White et al., 1987, Biochem. J. 247:23-28). EFEE to fluorescein conversion occurs via the specific cleavage of an ether linkage by a polycyclic aromatic hydrocarbon (PAH)-induced cP450 (Miller, A. G., 1983, Anal. Chem. 133:46-57). As with other cP450-catalyzed reactions, EFEE metabolism requires NADPH, and can be inhibited by carbon monoxide or monoclonal antibodies that decrease PAH or benzo(a)pyrene metabolism (Miller, A. G., 1983, Anal. Chem. 133:46-57). At 18 h prior to cP450 assay, cells were induced with 1 nM of a 1 .mu.M stock solution of the non-fluorescent compound, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Chemical Carcinogen Repository, National Cancer Institute, Kansas City, Mo.) in dimethylsulfoxide (DMSO) (Sigma Chem. Co.). Cultured cells were lifted using a dispase-collagenase mixture, pelleted and resuspended in phosphate buffered saline (PBS) at a density of .about.5.times.10.sup.5 cells/ml, stored on ice for 1 h, and gradually warmed to 37.degree. C. Cells were incubated with 50 nM EFEE (Molecular Probes, OR) in PBS for 5 min at 37.degree. C. and examined for green fluorescence on a flow cytometer with a 515 nm long-pass filter and tuned to the 488 nm band. Fluorescence resulting from EFEE to fluorescein conversion was gated on various populations of cells based on differences in FLS vs. SS characteristics and was measured once/minute for up to 25 min in samples maintained at 37.degree.C.

Detailed Description Text (94):
7. EXAMPLE: GENETICALLY-ENGINEERED LIVER CELL CULTURES

Other Reference Publication (10):
Leighton, J. et al., 1968, Cancer Res. 28:286-296.

Other Reference Publication (25):
Yang, J. et al., 1981, Cancer Res. 41:1021-1027.

Other Reference Publication (74):
Sirica, A., Hwang, C., Sattler G. and Pitot, H., 1980, Cancer Res. 40:3259-3267.

L28: Entry 490 of 570 File: USPT Dec 8, 1998

DOCUMENT-IDENTIFIER: US 5846796 A
TITLE: Blood-borne mesenchymal cells

Abstract Text (1):
The present invention relates to a population of blood borne mammalian cells that express a unique profile of surface markers that includes certain markers typical of connective tissue fibroblasts, and are referred to herein as "blood-borne mesenchymal cells." In particular, it relates to the isolation, characterization and uses of such blood-borne mesenchymal cells. The cells of the present invention can be distinguished from peripheral blood leukocytes by their distinct size, morphology, cell surface phenotype and biologic activities, and are likewise distinguishable from connective tissue fibroblasts by other surface phenotypic markers. These cells proliferate in culture, and in vivo, as demonstrated in animal models, are capable of migrating into wound sites from the blood. Therefore, such blood-borne mesenchymal cells may have a wide range of applications, including, but not limited to, the promotion of wound healing, tissue remodeling, and for gene therapy.

Brief Summary Text (24):
The present invention relates to a population of blood-borne mammalian cells that express a unique profile of surface markers that includes certain markers typical of connective tissue fibroblasts, and are referred to herein as "blood-borne mesenchymal cells." In particular, it relates to the isolation, characterization and uses of such blood-borne mesenchymal cells. The cells of the present invention can be distinguished from peripheral blood leukocytes by their distinct size, morphology, cell surface phenotype and biologic activities, and are likewise distinguishable from connective tissue fibroblasts by other surface phenotypic markers. These cells proliferate in culture, and in vivo, as demonstrated in animal models, are capable of migrating into wound sites from the blood. Therefore, such blood-borne mesenchymal cells may have a wide range of applications, including, but not limited to, the promotion of wound healing, tissue remodeling, and for gene therapy.

Brief Summary Text (29):
After the first 24 hours, additional cellular elements arrive and contribute to the wound healing process. Blood-borne neutrophils and monocytes migrate into the wound site. These cells function in part by neutralizing invading microorganisms and secreting enzymes that clear away the initial clot. During this second, often referred to as "inflammatory" phase of wound repair, macrophages play a primary role by secreting a variety of inflammatory cytokines such as tumor necrosis factor (TNF), the interleukins such as IL-1, IL-6, IL-8, transforming growth factor-.beta. (TGF-.beta.), etc., and growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), that serve to combat infection and recruit additional cell types. These cell types include the epithelial and connective tissue cells particularly fibroblasts, that ultimately repair the site of tissue damage. The final phase of tissue repair is tissue remodeling, involving collagen cross-linking, collagenolysis and collagen synthesis for increasing structural integrity within the wound. Unfortunately, this entire process takes a relatively long time to complete.

Brief Summary Text (32):
Over a period of days to weeks, tissue repair and remodeling processes continue to take place. In skin, epithelialization occurs as neighboring, epithelial cells grow into the wound site to protect it while the subjacent dermis is repaired. Connective tissue mesenchymal cells, also referred to as fibroblasts are the primary mediators of this later phase of wound healing. These cells proliferate within the wound site and produce collagens and other matrix components. During this phase, a cellular and macromolecular framework is established that is responsible for the ultimate reorganization of a particular tissue or organ. Smooth muscle cells and vascular endothelial cells also repopulate the wound site. New blood vessels form to support and nourish the newly established tissue.

Brief Summary Text (37):
A wide variety of uses of the blood-borne mesenchymal cells, and factors produced by these cells, are encompassed by the invention described herein, particularly to improve wound healing, including, but not limited to, cutaneous-wounds, corneal wounds, wounds of epithelial-lined organs, resulting from physical abrasions, cuts, burns, chronic ulcers, inflammatory conditions and the like, as well as from any surgical procedure. Alternatively, the mesenchymal cells may be genetically engineered to express one or more desired gene products. The engineered cells may then be administered in vivo (e.g., either returned to the autologous host or administered to an appropriate recipient) to deliver their gene products locally or systemically.

Detailed Description Text (2):
The present invention relates to mammalian blood-borne mesenchymal cells, to methods of isolating and characterizing the cells, and to methods of using the same for a variety of applications including but not limited to wound healing and gene therapy.

Detailed Description Text (6):
A variety of separation procedures may be employed for obtaining mesenchymal cells involved in wound healing from the peripheral blood. Variants of such methods which are illustrated in the working examples described in Section 6 are included within the scope of the present invention. In accordance with this aspect of the invention, blood-borne mesenchymal cells may be isolated by separation based on the presence or absence of specific cell surface markers. These techniques may include flow cytometry using a fluorescence activated cell sorter or biotin-avidin or biotin-streptavidin separations using biotin-conjugated to marker-specific polyclonal or monoclonal antibodies and avidin or streptavidin bound to a solid support such as affinity column matrix or plastic surfaces, magnetic separations using antibody-coated magnetic beads, destructive separations such as antibody plus complement or antibody coupled to cytotoxins or radioactive isotopes for the removal of undesirable cell populations.

Detailed Description Text (15):
The ability of the blood-borne mesenchymal cells to proliferate in culture indicates that they may be expanded in numbers for use in wound healing, or gene therapy applications, particularly in autologous and syngeneic hosts. To that end, the cells may be used directly after isolation, or after in vitro culture with or without the introduction of exogenous genes, and with or without expression in culture.

Detailed Description Text (17):
In general, genetic engineering of the cells involves isolating blood-borne mesenchymal cells from an individual, transferring a gene of interest into these cells, confirming stable integration and expression of the desired gene products. Such genetically engineered cells may be transplanted into the same, or an HLA-matched, or otherwise suitable patient and/or used as a source of factors and/or genes encoding factors made by the cells. For the practice of the invention,

mesenchymal cells isolated by the procedures described in Section 6, infra, may be used as recipients in gene transfer experiments. The cells may be grown in culture prior to, during, and after introduction of an exogenous gene. The proliferative activity of these cells may be enhanced by GM-CSF. For the introduction of exogenous genes into the cultured mesenchymal cells, any cloned gene may be transferred using conventional techniques, including, but not limited to, microinjection, transfection and transduction.

Detailed Description Text (19):
For long-term, high-yield production of recombinant proteins, stable expression is often preferred. Rather than using expression vectors which contain viral origins of replication, the mesenchymal cells can be transformed with a cDNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker confers resistance to the selection and allows cells to stably integrate the recombinant DNA into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered mesenchymal cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. U.S.A. 48: 2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22: 817) genes. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Proc. Natl. Acad. Sci. U.S.A. 77: 3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150: 1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30: 147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. U.S.A. 85: 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

Detailed Description Text (22):
Alternatively, the cells may be administered to patients via any of a number of routes, including but not limited to intravenous, intramuscular, subcutaneous, intradermal, etc., for the treatment of external or internal visceral injuries. Where the method of administration allows for cell migration (e.g., intravenous administration), the mesenchymal cells will migrate in vivo to the site of the wound where they can enhance wound healing. Genetically engineered mesenchymal cells may be used in this fashion to deliver gene products to the site of the wound; e.g., genes for Factor VIII, growth factors, etc. may be useful in this regard. Alternatively, methods of administration which do not allow for migration may allow the mesenchymal cells, genetically engineered or otherwise, to take up residence at the site of administration where they can deliver gene products to the local environment, and/or systemically.

Detailed Description Text (27):
Monoclonal antibodies to novel antigens on these mesenchymal cells may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256, 495-497), and the more recent human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci. 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and

Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Techniques developed for the production of "chimeric antibodies" by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule can be used (e.g., Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature 314:452-454). In addition, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce single chain antibodies.

Detailed Description Text (31):
In order to identify new cytokines that may be produced by the mesenchymal cells, long-term mesenchymal cell cultures may be established or continuous cell lines may be generated by transforming the cells to tumor cells using a virus or a chemical. Culture supernatants may be directly analyzed by applying them to various cell types or in various animal models, which can then be assayed for the appropriate desired biological response. The cells may be metabolically labelled and their supernatants subjected to biochemical analysis to identify candidate proteins responsible for the observed bioactivity. Additionally, cytokines may be identified by inducing cytokine production in the cells. To this end, the cells may be exposed or contacted with an agent that induces the expression and production of a cytokine. A number of agents known to induce cytokine production in other cells may be useful in this approach. Such agents may include but are not limited to calcium ionophores, endotoxins, phorbol esters, known cytokines, chemokines, growth factors, hormones and/or other mediators. Having identified a candidate protein by SDS-PAGE and/or by biologic activity, the protein may be purified by a variety of techniques known in the art including but not limited to SDS-preparative gels, ion exchange chromatography, isoelectric focusing gels and other types of chromatography. Purity of the proteins can be verified by SDS-PAGE, quantified by protein assays, their activities confirmed in bioassays, and used as immunogens for the production of polyclonal and monoclonal antibodies.

Other Reference Publication (7):
Anderson, W.F. Gene Therapy. Scientific American Sep. 1995 p. 128 col. 1 Lines 50-58.

CLAIMS:

1. An isolated population of mammalian blood-resident cells comprising a desired gene operably associated with a regulatory region that controls gene expression genetically engineered into the mammalian blood-resident cells, wherein the desired gene is expressed by the mammalian blood-resident cells, and wherein the mammalian blood-resident cells display surface phenotypic markers of fibroblasts and CD45 and CD34 phenotypic markers of hematopoietic stem cells.

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L28: Entry 511 of 570 File: USPT Jun 9, 1998

US-PAT-NO: 5763266
DOCUMENT-IDENTIFIER: US 5763266 A

TITLE: Methods, compositions and devices for maintaining and growing human stem and/or hematopoietics cells

DATE-ISSUED: June 9, 1998

US-CL-CURRENT: 435/289.1; 435/293.2, 435/297.2, 435/372, 435/402

APPL-NO: 08/ 290773 [PALM]
DATE FILED: November 7, 1994

PARENT-CASE:
CROSS-REFERENCE TO RELATED APPLICATIONS This application is a continuation application Ser. No. 07/845,969, filed Mar. 4, 1992, now abandoned, which is a continuation-in-part of application Ser. No. 07/366,639, filed Jun. 15, 1989, now abandoned.

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	APPL-DATE
WO	PCT/US93/01803	March 4, 1993

PCT-DATA:

APPL-NO	DATE-FILED	PUB-NO	PUB-DATE	371-DATE	102(E)-DATE
PCT/US93/01803	March 4, 1993	WO93/18132	Sep 16, 1993	Nov 7, 1994	Nov 7, 1994

L28: Entry 519 of 570 File: USPT Nov 11, 1997

DOCUMENT-IDENTIFIER: US 5686278 A
**** See image for Certificate of Correction ****
TITLE: Methods for enhanced retrovirus-mediated gene transfer

Abstract Text (1):
A method to increase the efficiency of transduction of hematopoietic cells by retroviruses includes infecting the cells in the presence of fibronectin or fibronectin fragments. The fibronectin and fibronectin fragments significantly enhance retroviral-mediated gene transfer into the hematopoietic cells, particularly including committed progenitors and primitive hematopoietic stem cells. The invention also provides improved methods for somatic gene therapy capitalizing on the enhanced gene transfer, and hematopoietic cellular populations.

Brief Summary Text (4):
Progress in understanding the molecular basis of many human diseases as well as improvement in gene transfer technology has led to recent attempts to develop protocols for somatic gene therapy for severe genetic diseases. Currently, promising disease candidates for human gene therapy include those in which an enzyme or other protein is defective or missing, where the level of enzyme or protein does not need to be exactly regulated, especially those that are constitutively regulated, and those defects which are found in the patient's bone marrow.

Brief Summary Text (5):
For example, one disease candidate for gene therapy is adenosine deaminase (ADA) deficiency which results in severe combined immunodeficiency disease (SCID). ADA deficient patients have little or no detectible enzyme in bone marrow cells. However, ADA deficiency has been cured by matched bone marrow transplantation. ADA normal cells have a selective advantage over ADA deficient cells and will normally repopulate the patient's bone marrow.

Brief Summary Text (6):
Bone marrow cells are a good target for somatic gene therapy because bone marrow tissue is easily manipulated in vitro and contains repopulating cells. Alternatively, human cord blood has previously also been demonstrated to contain a large number of primitive progenitor cells. Successful gene transfer into hematopoietic stem cells, the long term repopulating cells, may lead to lifelong cures for a variety of diseases manifested in the progeny of these cells.

Brief Summary Text (8):
In both murine and large animal experiments involving bone marrow cells, it has been noted that the most successful protocols utilize cocultivation of target cells with retroviral producer cell lines. Also, most of the FDA-approved gene transfer trials in humans rely on recombinant retroviral vectors for gene transduction. Recombinant retroviral vectors are desireable for gene therapy because they efficiently transfer and precisely and stably integrate exogenous DNA into cellular DNA. These vectors contain exogenous DNA for gene transfer and are further modified to eliminate viral pathogenicity. Because of these modifications, viral production is generally accomplished using retrovirus packaging cells. However, for clinical

gene therapy, cell-free transduction is more desirable due to concerns about bio-safety and quality control. Unfortunately, efficient gene transfer into hematopoietic cells such as stem cells has generally not been possible without cocultivation with virus-producing cells.

Brief Summary Text (9):
Recently, it has been shown that gene transfer efficiency can be increased by exposing target cells to stromal cells during infection. Stromal cells are a major component of the hematopoietic microenvironment (HM). The HM consists of an organized network of macrophages, stromal cells, endothelial cells, adipocytes and a complex extracellular matrix made up of a variety of defined adhesion molecules. ECM molecules such as laminin, collagen, thrombospondin, proteoglycans, glycosaminoglycans and fibronectin provide anchorage sites for both hematopoietic cells and growth factors. The mechanism underlying this promoting effect of stroma on retroviral infection is unclear, but it has been known for some time that physiologic regulation of the proliferation and differentiation of hematopoietic cells occurs when these cells are in direct contact with cells of the HM.

Detailed Description Text (6):
Retroviral vectors that include exogenous DNA providing a selectable marker or other selectable advantage are preferred. For example, the vectors can contain one or more exogenous genes that provide resistance to various selection agents including antibiotics such as neomycin. Representative vectors which can be used in the invention include the N.sub.2 /ZipTKNEO vector (TKNEO) (titer: 1.times.10.sup.5 G418.sup.r cfu/ml on NIH 3T3 cells), the ZipPGK-hADA vector, and the ZipPGK-mADA vector all as previously reported by Moritz et al. (1993) J. Exp. Med. 178:529. In the TKNEO vector, neo phosphotransferase sequences are expressed in the sense orientation (relative to the 5' long terminal repeat-LTR) via the herpes simplex thymidine kinase promoter. This vector contains a selectable maker gene which provides neomycin resistance to facilitate the identification of transduced cells. In the ZipPGK-hADA vector, the hADA cDNA is expressed in the sense orientation relative to the 5'LTR via the human phosphoglycerate kinase (PGK) promoter. It contains only one expressable genetic sequence and lacks a dominant selectable marker. The ZipPGK-mADA (PGK-mADA) vector is identical to the ZipPGK-hADA vector except the human ADA cDNA has been replaced with murine ADA DNA. These and other retrovirus vectors and techniques for their production are well known and their implementation and use in the present invention will be well within the skills of those practiced in the art given the disclosure herein.

Detailed Description Text (9):
One aspect of the invention provides a method of somatic gene therapy which involves in-vitro cellular therapy and transplantation into a host. Hematopoietic cells can be collected from a human or other mammalian or animal source using standard protocols. For example, the hematopoietic cells can be collected from donor bone marrow or peripheral blood or from animal, e.g. human, fetal cord blood. Once collected, the hematopoietic cells can optionally be treated so as to enrich them in stem cells and/or primitive progenitor cells. The hematopoietic cells can then be suitably incubated, for instance on tissue culture plates. Optionally during this period, adherent-negative low density mononuclear cells can be prestimulated prior to retroviral infection. Prestimulation as used herein refers to the process of exposing cells to growth stimulating factors before exposure to retroviruses.

Detailed Description Text (11):
Methods of the invention can be used in gene marking or gene therapy protocols for a variety of bone marrow disorders, including for example cancers, leukemias, disorders involving protein deficiencies or abnormalities, and for modifying hematopoietic cells to improve resistance to other therapeutic protocols such as chemotherapy. Representative disorders with which the invention may be used thus include ADA deficiency, e.g. ADA-deficient SCID, pediatric acute myelogenous

leukemia (AML), neuroblastoma, and adult AML and acute lymphocytic leukemia (ALL).

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L30: Entry 12 of 17 File: PGPB Feb 13, 2003

DOCUMENT-IDENTIFIER: US 20030031660 A1
TITLE: Method of inducing bone formation

Summary of Invention Paragraph:
[0009] The use of marrow stromal cells for cell and gene therapy: Bone arrow stromal cells (MSCs) have the potential to differentiate into a variety of mesenchymal cells. Within the past several years MSCs have been explored as vehicles for both cell and gene therapy. These cells are relatively easy to isolate from small aspirates of bone marrow that can be obtained under local anesthesia; they are also relatively easy to expand in culture and to transfect with exogenous genes. Several different strategies are being pursued for the therapeutic use of MSCs as follows: